Effect of olanzapine on feeding and selected biochemical factors related to weight gain

A thesis submitted to the College of Graduate Studies and Research

in partial fulfillment of the requirements

for a Master's degree

in the Department of Psychiatry

University of Saskatchewan

Saskatoon

By

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Abstract

Olanzapine is an atypical antipsychotic drug exhibiting a low incidence of extrapyramidal side effects. It is not only effective in treating positive symptoms of schizophrenia, but also more efficacious against negative and depressive symptoms than classical antipsychotics. Olanzapine has been recommended as the first-line drug for the treatment of schizophrenia. Unfortunately, a common side effect of olanzapine, namely weight gain, has also been observed. A comprehensive literature analysis revealed that olanzapine induced higher weight gain than most other antipsychotics, only second to clozapine. The incidence of olanzapine-induced weight gain and related diseases, such as diabetes and cardiovascular diseases, is higher than that of the general population. These unwanted side effects have decreased the adherence to treatment. Many clinical observations and preliminary animal studies have attempted to elucidate the possible mechanism involved. To date, the mechanism for olanzapine-induced weight gain remains unclear.

This research project utilizes an animal model to investigate the possible mechanism of olanzapine-induced weight gain. The specific research objectives include: 1) does olanzapine affect feeding behavior; 2) can olanzapine influence the levels of glucose and triglyceride; 3) are cytokines, such as insulin, leptin, and TNF-α involved in olanzapine-induced weight gain; 4) how does olanzapine affect adipose tissue?

An olanzapine-induced weight gain animal model has been established in the present investigation. An increase in food and water intake and increase in fat deposition accompanied with weight gain after treatment were observed. No significant increase in levels of glucose and triglyceride was detected. The changes of insulin and leptin levels

in blood suggest that olanzapine may affect the endocrine system. A dramatic morphological alteration of adipose tissue by olanzapine was serendipitously observed. Immunohistochemical staining revealed that olanzapine stimulated collagen VI expression and deposition in the extracellular matrix suggesting that adipocyte differentiation may be enhanced. The effect of olanzapine on fat deposition might play a critical role in olanzapine-induced weight gain. The data from adipose tissue have provided a new clue on future research in understanding the mechanism of olanzapine-induced weight gain. Due to limitation of small number of animals and relatively short term of treatment, a large variation in groups diminished the power of analysis regarding the effects of olanzapine related to weigh gain.

Acknowledgements

There are a number of people that I would like to thank who have supported me during my education and who have helped me complete my dissertation.

I would like to thank my supervisor, Dr. Peter H.Yu. He made my work possible and provided me with the environment, and resources to learn. I thank him for his continued guidance and patience during the last three years. Thanks are also extended to the other members of my advisory committee: Dr. Xin-Min Li, and Dr. Darrell D. Mousseau for their constructive guidance and kind support.

I also thank Eli Lilly Company for the kind supply of olanzapine for this study.

I would like to give thanks to many of the staff, post-doctoral fellows and students of the Neuropsychiatry Research Unit and of the other departments who supplied assistance in my program, especially Dr. H. Fan and L.X. Lu for their kind assistance in caring about the protein profile analysis and cell culture experiments, Dr. M. Kazachkov for HPLC, G.J. Wei for establishing ELISA method. Many people have been very helpful for giving both moral and technique support, especially Bruna Bonavia-Fisher, Li-xin Lu, and Ze-lan Wei.

Lastly, I must thank my mother and father for their outstanding kindness, patience, encouragement and support they have given me throughout my university studies. I especially thank my father for leading my interests to science and sharing the happiness from research. I would also thank my husband Wentong for being patient, understanding and moral support during the preparation for the thesis.

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List of abbreviations

AgRP	agouti related protein
APS	adapter molecule containing PH and SH2 domains
BSA	bovine serum albumin
BMI	body mass index
CPT-1	carnitine palmitoyl transferase-1
CNS	central nervous system
DA	dopamine
Dex	dexamethasone
ELISA	enzyme-linked immunosorbent assay
EPS	extrapyramidal side effects
FATP	fatty acid transporter
FFA	free fatty acid
GK	glycerol kinase
Glut4	glucose transporter 4
G-1-PDH	glycerol -1- phosphate dehydrogenase
G-1-P	glycerol-1-phosphate
Gab	GRB2- associated binder
H_1	histamine
HDL	high density lipoprotein
HPLC	high Performance Liquid Chromatography
5-HT	5-Hydroxytryptamine (Serotonin)
IBMX	isobutylmethylxanthine
IL-6	interleukin-6
INT	2-(p-iodophenyl)-3-p-nitrophenyl-5-phenyltetrazolium chloride
INTH	formazan
IR	insulin receptor
IRS	insulin receptor substrate
JAK	janus kinase
LSD	lysergic acid diethylamide
α-MSH	α-melanocyte stimulating hormone

MTT	3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide
NPY	neuropeptide Y
PBS	phosphate Buffered Saline
PI3K	ptdIns 3-kinase
PKC	protein kinase C
POMC	proopiomelanocortin
PPAR	peroxisome proliferator activated receptor
Shc	Src-homology and collagen homology
SOCS-3	suppressor of cytokine signalling protein-3
STAT3	signal transducer and activator of transcription-3
SREBP	sterol regulatory element binding protein
TNF-α	tumor necrosis factor α
TMB	tetramethylbenzidine dihydrochloride
TNFR	tumor necrosis factor α receptor
UCPs	uncoupling proteins

1. Introduction

1.1 Schizophrenia and antipsychotics

Schizophrenia is a major psychiatric illness. The symptoms of this disease include positive and negative features. Delusion, hallucinations and thought disorder are positive symptoms; negative symptoms include withdrawal and flattening of emotional responses. The cause of schizophrenia is still unclear. Few animal models have been established for schizophrenia research. Indirect evidence from human and experimental animals suggests that neurochemicals in the brain are involved in the symptoms of schizophrenia (Rong et al, 2003).

The dopamine hypothesis of schizophrenia was proposed in 1965. Amphetamine, a chemical capable of releasing dopamine in the brain, can produce acute schizophrenic-like behaviour symptoms in human, and can also exacerbate schizophrenic symptoms. In animals, dopamine-releasing agents cause a type of stereotypic behaviour resembling the repetitive behaviours seen in schizophrenic patients. Dopamine receptor agonists exhibit similar effects in animals. Dopamine antagonists and drugs that block neuronal dopamine storage can control positive symptoms of schizophrenia and amphetamine-induced behaviour changes. The antipsychotic efficacy of dopamine antagonists is correlated with their activity in blocking the dopamine D_2 receptors (Strange, 2001).

Several neurotransmitters, especially those interacting strongly with dopamine pathways, such as 5-HT, norepinephrine and glutamate, seem also to be involved in the etiology of schizophrenia. The hypothesis that 5-HT dysfunction may be involved in schizophrenia is based on the observation that lysergic acid diethylamide (LSD) also produces schizophrenic-like symptoms. The main behavioural effect caused by LSD is

hallucinations. As a 5-HT agonist, LSD mainly acts on 5-HT_2 receptors in the CNS. 5-HT has a modulatory effect on dopamine pathways. Many so-called "atypical" antipsychotic drugs work as antagonists both on 5-HT and dopamine receptors. They produce fewer extrapyramidal side effects (EPS) than those drugs that mainly targeting the dopaminergic system. It was postulated that blocking 5-HT_2 receptors may counteract the undesirable side effects related to D_2 receptor antagonists (Busatto et al., 1997).

The neurochemical hypothesis of schizophrenia is supported by indirect evidence. Although it is oversimplified, it provides fundamental knowledge for understanding the action of antipsychotic drugs.

Antipsychotic drugs available have been classified as "typical" or "atypical". These terms are not clearly defined. Generally, the distinction rests on the incidence of extrapyramidal side effects, efficacy in treatment–resistant groups of patients, and efficacy against negative symptoms.

All antipsychotic drugs exhibit antagonistic effects on D_2 receptors. Clinical efficacy of antipsychotic agents is achieved when approximately 80% of D_2 receptors are occupied. Blocking function of D_2 receptor by antipsychotics can be measured by *in vitro* experiments, i.e., assessment of the ability to inhibit the binding of a radioactive D_2 antagonist to brain membrane fragments. The effect of D_2 antagonists can also be measured by assessing the inhibition of amphetamine-induced stereotypic behaviour in animals. Different types of drugs show different affinities to dopamine receptors. Some are highly selective for D_2 receptors; some are relatively non-selective between D_1 and D_2 (Seeman, 1987).

Antipsychotic drugs cause several types of unwanted effects related to their pharmacological profiles. Due to the blockade of D_2 receptors, EPS are the most common side effects. The effects include movement disorders, e.g. acute dystonias and tardive dyskinesia. Acute dystonias are involuntary movements, tremor and rigidity. They occur in the first few weeks and can be reversed by stopping treatment. Tardive dyskinesia develops after months or years in 20-40% of patients treated with typical antipsychotic drugs. This is a serious side effect since it is irreversible, untreatable, and can worsen when the antipsychotic-treatment is stopped, which can disable the patient. The syndrome comprises involuntary movement of face and tongue, and also of the trunk and limbs. The occurrence of this syndrome depends on the duration and dosages of the drugs, and the age of the patients (Klawans et al., 1988). One hypothesis about tardive dyskinesia is that it is related to gradual increase in the density of D_2 receptors *via* up-regulation in the striatum. Another theory is that chronic inhibition of dopamine receptors enhances catecholamine and/or glutamate release in the striatum. Glutamate has been reported to cause excitotoxic neurodegeneration (Casey, 1995). Other common side effects caused by antipsychotic drugs include sedation, hypotension and weight gain.

Antipsychotic drugs can improve the acute symptoms of schizophrenic patients and long-term treatment can prevent the recurrence of symptoms and lead to a more normal life of the patients. However, it has been found that typical antipsychotics are only effective in about 60%-70% of patients. Those patients who do not respond to drugs are known as “antipsychotic-resistant”. Although these drugs are efficient in controlling positive symptoms, they are not effective in relieving negative symptoms. Compared to typical antipsychotics, atypical drugs are capable of overcoming, to some degree, these

shortcomings. They are not only effective in treating positive symptoms of some "antipsychotic-resistant" patients, but also improve negative symptoms, as well as decreasing the risk of EPS and tardive dyskinesia dramatically (Rang et al, 2003).

1.2 Atypical antipsychotics and weight gain

It has been reported that atypical antipsychotics are capable of causing weight gain, even obesity, in patients in comparsion to typical antipsychotics. Obesity and weight gain are well known risk factors for hypertension, type II diabetes, coronary heart disease, stroke, gallbladder disease, respiratory problems and even some types of cancer in general population (Allison et al., 1999). Indeed, it has been observed that atypical antipsychotic-treated obese patients are more likely to develop type-II diabetes and cardiovascular diseases. Severe weight gain and obesity-related diseases cause patients to discontinue their treatment. A retrospective analysis showed that the degree of weight gain ranged from 0.04 kg for ziprasidone to 4.45 kg for clozapine, estimated by the random effects regression at 10 weeks (figure1.2.1). This data clearly indicated that atypical antipsychotics might have more tendency to cause weight gain than most of the classical antipsychotics (Allison et al, 1999).

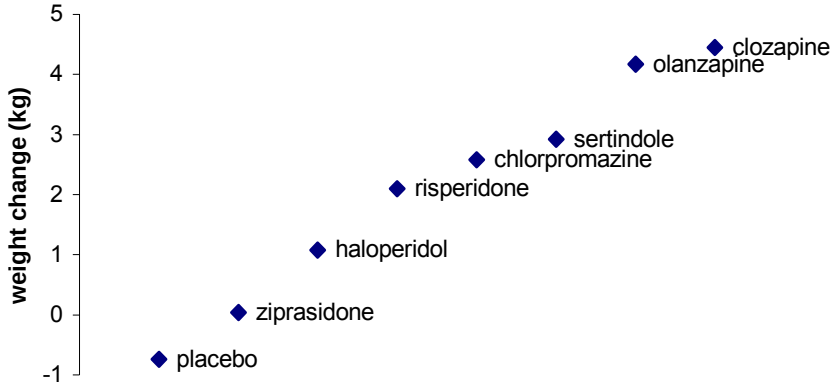


Figure 1.2.1 Weight change induced by antipsychotics after 10 weeks on standard drug doses, estimated from a random effect model. (Data was adapted from Allison et al., 1999)

Regarding antipsychotic-induced weight gain, a positive correlation between body weight gain and the dosage of clozapine has been reported. A dose-dependent effect of olanzapine on weight gain has also been observed (Russell et al., 2001). Other potential factors related to antipsychotic-induced weight change have been noticed, such as patients with low pre-treatment body mass index, young age, certain psychiatric disorders or using mood stabilizers. The association between weigh gain and these factors needs further studies (Russell et al., 2001). Some adverse metabolic effects of antipsychotics have also been observed in overweight patients, including glucose and lipid abnormalities, hyperinsulinaemia – indicating insulin resistance, and hyperleptinaemia (Melkersson et al., 2004; Meyer, 2001; Russell et al., 2001).

The mechanism of antipsychotic-induced weight gain is still unclear. Some hypotheses focus on the neurotransmitters related to feeding behaviour and weight regulation. Dopamine and serotonin can decrease feeding behaviour in animal models, the serotonin antagonists stimulate food intake (McIntyre et al., 2001a). Histamine receptors are involved in drinking behaviour in rats. It is postulated that blocking the receptors of these neurotransmitters by antipsychotics may be involved in the mechanism of weight gain (McIntyre et al., 2001b; Zimmermann et al., 2003). However, an analysis of serotonin receptors failed to establish a correlation between weight gain induced by clozapine, olanzapine, risperidone, haloperidol, and sertindole with 5-HT_{2C} receptor affinity (McIntyre et al., 2001a).

Severe weight gain induced by antipsychotics also changes the levels of hormones related to weight regulation, such as insulin and leptin. The change in hormones may affect the sensitivity of satiety neurons in the lateral hypothalamus, and metabolism in the human body (Friedman, 1998). The hyperinsulinemia observed in antipsychotic-treated patients may lead to hyperlipidaemia. Hyperglycaemia and diabetes will develop due to insulin resistance. Leptin, the hormone produced by adipocytes, suppresses appetite through its receptor in the hypothalamus, and also regulates lipid metabolism in peripheral tissue. Hyperleptinaemia in antipsychotic-treated patients may indicate leptin resistance. This resistance may result in an increase in appetite and lipid abnormality, exacerbating weight gain (Melkersson et al., 2004). These hypotheses need further study.

1.3 Olanzapine

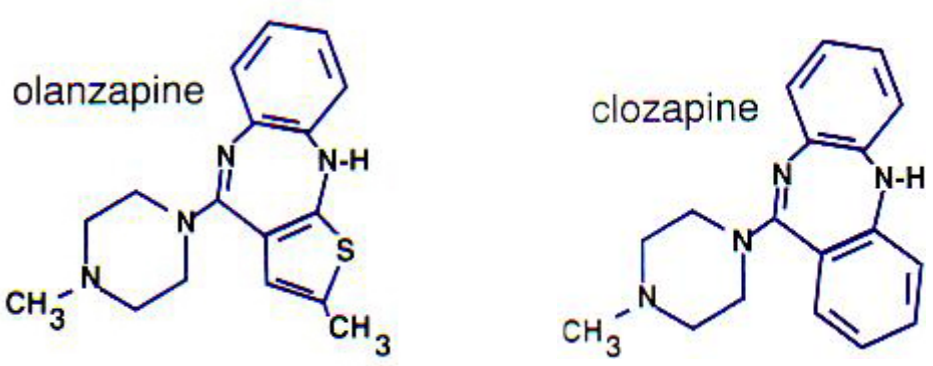


Figure 1.3.1 The chemical structures of olanzapine and clozapine

Olanzapine is a thienobenzodiazepine. Its structure is similar as that of clozapine, which is the archetypal atypical antipsychotic (figure 1.3.1) (Green, 1999; Trevitt et al., 1999). Experiments *in vitro* have shown that olanzapine affects key receptors, which are believed to be related to schizophrenia. The binding profile indicates that olanzapine has high affinity for 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C} and 5-HT_6, and histamine (H_1) receptors, as well as a moderate affinity for D_2 and acetylcholine muscarinic receptors, and a low affinity for β-adrenergic receptors (Stephenson et al., 1999; Trevitt et al., 1999). Olanzapine also interacts non-selectively with other dopamine receptors and 5-HT_1, 5-HT_3, 5-HT_4 and 5-HT_7 receptors. The limbic selectivity of olanzapine reflects its atypical antipsychotic properties. *c-fos* is an early oncogene and its expression was used as a marker for neuronal activity. Olanzapine induces *c-fos* expression in the nucleus accumbens, which also can be increased by other atypical antipsychotics, and usually correlates with efficacy in treating positive symptoms. Olanzapine also induces *c-fos* in the prefrontal cortex, which might be related to its efficacy in treating negative

symptoms. These effects are similar to what has been observed with clozapine (Stephenson et al., 1999).

Behavioural and pharmacological studies of olanzapine confirm that it interacts with dopamine, serotonin and muscarinic receptors, and exhibits clozapine-like profiles, including mesolimbic selectivity, blocking 5-HT receptors at a lower doses than that for dopamine receptors, and inhibiting the conditioned avoidance response (indication of antipsychotic efficacy) at lower dose than that required to induce catalepsy (indication of EPS) (Moore et al., 1997). Double blind clinical studies show that olanzapine is highly efficacious in treating psychotic symptoms, while it has low liability to induce tardive dyskinesia or parkinsonism (Trevitt et al., 1999). In contrast to clozapine, olanzapine does not cause agranulocytosis in patients. Although clozapine has clear advantages over traditional antipsychotics, and causes less extrapyramidal side effects in patients, the agranulocytosis can be very serious and could be life threatening. Therefore, olanzapine has been considered as the first line drug and widely used in treatment of psychosis (Green, 2000; Stephenson et al., 1999).

Unfortunately, olanzapine also exhibits a common unwanted side effect, namely, increase in weight. Several clinical reports have shown that olanzapine-induced weight gain is accompanied with increased body fat, serum levels of glucose, triglyceride, insulin and leptin (Eder et al., 2001; Melkersson et al., 2000; Osser et al., 1999). Allison's report show that among different atypical antipsychotics, olanzapine cause the second highest degree of weight gain at 10 weeks using a random effects model, only lower than the weight gain caused by clozapine (Allison et al., 1999). Based on the definition from Federal Drug and Food Administration (U.S.A) that weight gain induced by any given

drug is an increase in body weight of more than 7% before treatment, a study showed that 29% of patients with olanzapine obtained 7% or more increase in body weight, while only 25% of patients treated with quetiapine, 18% with risperidone, and 9.8% with ziprasidone did (Muller et al., 2004). Investigations also show that olanzapine significantly increases the risk of developing diabetes over conventional antipsychotics (Koro et al., 2002). McIntyre and colleagues reported that Zolar retrospectively assessed 396 antipsychotic-treated patients and the result indicated that olanzapine tends to cause a higher incidence of type II diabetes than other antipsychotics. In this report, the prevalence of type II diabetes in the general population is 5%-7%. However, it was estimated that the incidence of type II diabetes caused by olanzapine was 11%, while 6.6% by haloperdol, 6% by risperidone, and 4.5% by fluphenazine (McIntyre et al., 2001b). Excess weight gain and obesity in these patients would increase the risk of hypertension, chronic cardiovascular disease and cancer. This side effect also causes distress in patients, affects their self-esteem and decreases treatment adherence.

1.4 Obesity

Obesity is defined as the state of severe increase in body weight, more specially adipose tissue, which impairs the health of individuals. A simple method to measure obesity is to calculate the *body mass index* (BMI). It is described as the weight in kilograms divided by the square of the height in metres (kg/m^2). The World Health Organization (WHO) defines the classification of BMI (table1.4.1) (Leong et al., 1999; Spiegelman et al., 2001). It has been reported that one-third of American people are obese with BMI more than 25 kg/m^2. Obesity has become a worldwide health problem,

and is a well-known risk factor for cardiovascular, respiratory, gastrointestinal disorders, diabetes and some types of cancer (McIntyre et al., 2001a; Spiegelman et al., 2001).

Table 1.4.1 The classification of body mass index (BMI)

Classification	Body Mass Index
Normal	18.5-24.9
Pre-obese (over-weight)	25.0-29.9
Obese, class 1	30.0-34.9
Obese, class 2	35.0-39.9
Obese, class 3	≥ 40.0

(table was adapted from Leong et al., 1999)

Body weight is maintained by the balance of energy intake and energy expenditure. A person gains weight when he is in positive energy balance (figure 1.4.1). The mechanisms of regulating in energy balance and body weight include control of the central nervous system (CNS), neuropeptides and cytokines (McIntyre et al, 2001; Spiegelman et al., 2001).

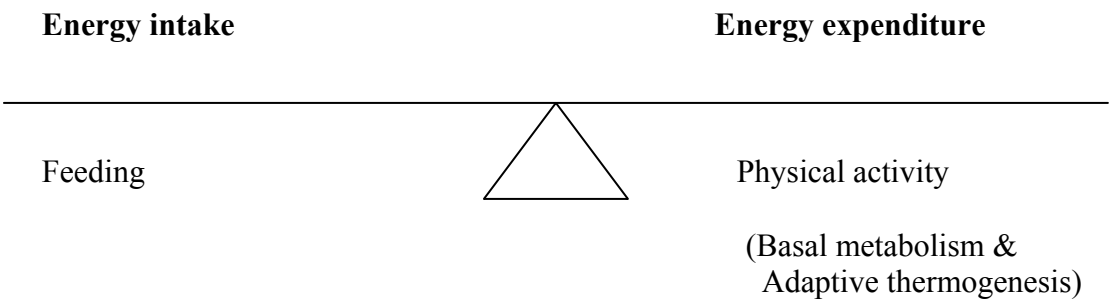


Figure 1.4.1 Component of the energy balance system (figure was adapted from Spiegelman et al., 2001).

The CNS influences energy balance by affecting feeding and physical behaviour and by affecting the neuroendocrine system. The hypothalamus is a critical region of the

brain related to the regulation of food intake. The monoamine activity in this region is thought to be responsible for the regulation. Dopamine decreases feeding behaviour in animal models (Floris et al**.,** 2001). Serotonin also decreases food intake, suggesting that serotonin antagonists stimulate energy intake. 5-HT_{2C} receptor-deficient mice have been shown to be overweight and to overeat (Tecott et al., 1995). Histamine (H_1, H_2, H_3) receptors in the hypothalamus are well known to be involved in mediating drinking behaviour. Histamine antagonism increases weight by stimulating appetite (McIntyre et al., 2001b). Neuropeptides and cytokines have also been shown to affect weight regulation. These peptides, such as insulin, leptin and tumor necrosis factor α (TNF-α), have been demonstrated to be involved in the regulation of glucose and general metabolism (McIntyre et al., 2001a).

1.4.1 Insulin

Insulin is the most potent hormone in promoting synthesis and storage of carbohydrates, lipids and proteins; and in inhibiting their degradation in adipose tissue, liver and skeletal muscle. Plasma insulin levels rise after eating. It stimulates glucose uptake and conversion to glycogen and/or triglycerides in muscle and adipose tissue. While insulin-mediated glycogen synthesis is increased in muscle and adipose tissue, insulin-dependent gluconeogenesis and glycogenolysis are suppressed in liver. Insulin also increases lipoprotein lipase activity in adipose tissue to clear the triglyceride-rich lipoproteins in plasma and inhibits adipocyte hormone-sensitive lipase activity to dramatically reduce the release of free fatty acids from adipose tissue. Insulin is the only known major hormone that promotes energy storage and inhibits energy utilization (Ruan et al., 2003; Saltiel et al., 2002).

Saltiel and colleagues (Saltiel et al., 2002) reviewed insulin signal pathway. Insulin's action on glucose metabolism is mediated by initiating the activity of the insulin receptor (IR), a tyrosine kinase. After insulin binds, the receptor undergoes autophosphorylation. With the increase in kinase activity, it subsequently phosphorylates several substrates, including the insulin receptor substrate (IRS), Gab-1, Shc, Cbl and adapter molecule containing PH and SH2 domains (APS), leading to activation of different pathways. Regarding the regulation of glucose transport, there are at least two signalling pathway models. One pathway that is initiated by IRS is PtdIns 3-kinase (PI3K) dependent. This kinase triggers a cascade of phosphorylation events. Phosphorylated IRS initiate a cascade of the PI3K pathway, including the activation of Akt and/or protein kinase C (PKC). Subsequently, the activated glucose transporter 4 (Glut4), phosphorylated by activated Akt and PKC, is translocated to the plasma membrane and promotes glucose transport into the cell. Insulin may also stimulate the cytoskeletal rearrangement for Glut4 location. Insulin receptor also phosphorylates Cbl and APS, which trigger a PI3K independent pathway to regulate glucose transport (Saltiel et al., 2002). Therefore, any factor influencing the sensitivity of the insulin receptor will affect the metabolism of glucose.

Insulin also affects insulin-sensitive enzymes, which are involved in glucose metabolism. Insulin increases the concentration of enzymes for glycolysis, such as hexokinase, phosphofructokinase-1 and pyruvate kinase. Insulin affects adipose cells by controlling the level of fatty acids by storing and hydrolysing triglyceride. It has been shown recently that insulin may partly regulate this process in promoting intracellular fatty acid transporters FATP1 and FATP4 to the plasma membrane. This raises the

intracellular fatty acids that are in turn converted to fatty acyl CoA. Insulin also increases lipoprotein lipase activity, resulting in the release of fatty acid from dietary fat to adipocytes, decreases to lipolysis of triglycerides stored in fat cells, and increases the esterification of fatty acids to triglycerides (Czech, 2002).

Insulin receptors not only exist in peripheral target tissues, but are also found in the hypothalamus. The PI3K pathway in the hypothalamus is activated after intracerebroventricular injections of insulin (Niswender et al., 2003a). Food intake-lowering effects of insulin are reversed by intracerebroventricular infusion of PI3K inhibitors, thus supporting a CNS role for insulin in the regulation of feeding behaviour (Niswender et al., 2003a; Niswender et al., 2003b).

Insulin resistance has been observed in human forms of obesity and diabetes. A hypothesis of insulin resistance is related to the high fat feeding behaviour. Intracellular insulin signalling pathway has been found to be impaired in high fat feeding models and intravenous lipid infusion models. Increased intracellular fatty acids may activate a serine/threonine kinase, but not tyrosine kinase, cascade, leading to the phosphorylation of serine/threonine sites on IRS. Serine-phosphorylation reduces the ability of IRS to interact with PI3K, thus failing to activate glucose transport and other downstream events (Niswender et al., 2003b; Shulman, 2000). As a consequence, cells become resistant or lose the response to the action of insulin even though enough insulin is produced. Higher levels of insulin are therefore required to regulate glucose metabolism. This resistance occurs often in people with obesity, type II diabetes, or high levels of fat in the blood.

1.4.2 Leptin

Leptin is an adipocyte-derived protein. It was first cloned in *ob*/*ob* mouse that have genetic deficiency of leptin genes. Leptin can exert satiety effects by limiting feeding behaviour and increasing energy expenditure. It is mainly synthesized in white adipose tissue. Chronic central leptin infusion in rats has been found to decrease food intake and as the result the body weight of these animals gradually decreased (Auwerx et al., 1998).

Leptin achieves its metabolic function through specific receptors located in the hypothalamus, the feeding centre, and in peripheral tissues, notably adipose tissue, liver, skeletal muscle and kidney (Auwerx et al., 1998; Cohen et al., 1996; Muoio et al., 2002). The receptors transmit the leptin signal to activate Janus kinase (JAK), a signal transducer and activator of transcription-3 (STAT3)-dependent pathway (Vaisse et al., 1996; Zabeau et al., 2003). The central leptin receptors bind leptin which is transported in blood and crosses the blood-brain barrier by a saturable transport system. Studies on leptin receptor signalling through the JAK-STAT3 signal pathway demonstrated that the phosphorylated leptin receptors and phosphorylated STAT3 remained elevated in the hypothalamus during chronic central leptin infusion, initiating a decrease in food intake (Pal et al., 2003). Several candidate effectors related with feeding behaviour and energy metabolism are thought to be regulated by leptin. The hypothalamic arcuate nucleus contains two primary types of neurons which express leptin receptors. Leptin activates the proopiomelanocortin (POMC) neuron to secret an anorexic neuropeptide, α-melanocyte stimulating hormone (α-MSH), resulting in reduction of food intake and an increase in energy expenditure. In contrast, leptin also reduces the food intake by

suppressing the secretion of the neuropeptide Y (NPY)/agouti related protein (AgRP) in neurons which potently stimulate food intake (Auwerx et al., 1998; Niswender et al., 2003b).

Leptin also affects lipid metabolism related to the activation of the JAK/STAT signal pathway in the peripheral system, such as adipose tissue, liver or skeletal muscle. Several downstream target including SREBP1, PPARγ and PPARα, are regulated. In liver, leptin suppresses mRNA expression of SREBP1 and PPARγ, thereby blocking their transcriptional activation involved in cholesterol synthesis, and also blocking lipogenesis and glycerolipid synthesis. Leptin increases the expression of PPARα, an activator of fatty acid oxidation and uncoupling proteins (UCPs). The increase in UCP activity can promote thermogenesis and decrease cellular levels of ATP (Muoio et al., 2002). Leptin stimulates the rate of lipolysis and increases lipid oxidation, i.e. increasing the expression of enzymes of fatty acid oxidation, such as carnitine palmitoyl transferase-1 (CPT-1) and acyl CoA synthase (Wang et al., 1999). Leptin also inhibits the activity of acetyl-CoA carboxylase, which is a rate-limiting enzyme in fatty acid synthesis. All these effects of leptin result in reducing intercellular fatty acid and triglyceride concentrations (Auwerx et al., 1998; Palou et al., 2000).

Both leptin and insulin exert critical effects on glucose and fatty acid metabolism. Although these two cytokines, and the receptors they act on, are totally different, evidence suggests that there is a cross talk between these two factors. With the infusion into brain, leptin, which acts through the JAK-STAT signal pathway, also increases PI3-Kinase activity (Zhao et al., 2002). Infusion of an inhibitor of this enzyme can block leptin-induced anorexia (Niswender et al., 2001). Leptin is also capable of activating the

IRS-PI3K pathway in adipose tissue and liver to exert insulin-like function to some degree (Kim et al., 2000). The cross-talk between insulin and leptin in the CNS and peripheral tissue cells indicate that factors, which affect PI3K pathway related to insulin and leptin, may alter feeding behaviour and nutrient metabolism.

Since leptin regulates feeding behaviour and energy balance, it becomes very important in the study of obesity. It was thought that deficiencies in leptin production or leptin receptor in *ob* mice might also be found in obese humans. However, neither deficiencies in leptin production nor mutations in the leptin gene or the leptin receptor gene were detected in most obese humans (Palou et al., 2000). Therefore, the alterations in the transport of leptin to the CNS or leptin resistance may take place in obesity. High serum leptin levels in obese humans are not matched by the levels of leptin in the cerebrospinal fluid, suggesting that a decrease in leptin transport may occur. Meanwhile, since both insulin and leptin can active the PI3K pathway, and leptin-reduced food intake is PI3K dependent, it is hypothesized that the mechanism of leptin resistance, or hyperleptinemia in human obesity might be similar to that of insulin resistance caused by uncorrected phosphorylation of PI3K (Niswender et al., 2001).

1.4.3 Tumor necrosis factor-α (TNF-α)

TNFα was first identified in the serum of endotoxin-treated mice and is responsible for the hemorrhagic necrosis of tumors (Argiles et al., 1997; Old., 1985). A 26 KDa membrane-bound monomer precursor protein cleaved by TNF-α converting enzyme to form mature secreted TNF-α trimer. Two TNF-α receptors, type I (p55), a 55-KDa protein, and type II (p75), a 75-KDa protein, have been identified. They mediate TNF-α signal transduction by forming protein complexes with cytoplasmic adaptor

proteins (Ruan et al., 2003). TNF-α not only exerts a variety of effects on growth promotion, growth inhibition, cytotoxicity and inflammation, but also plays a critical role in regulation of metabolism, immune responses and appetite behaviours (Aggarwal et al., 1996). Different cell types, such as macrophages and monocytes, adipocytes and muscle cells express TNF-α(Saghizadeh et al., 1996).

Studies have indicated that there is a link between TNF-α and obesity. mRNA expression and production of TNF-α protein are increased in adipose tissue of obese humans. The plasma concentration of soluble TNF-α receptors (TNFR) was also found to be increased in obese subjects. Body weight reduction in these subjects is accompanied with a decrease in mRNA expression of TNF-α (Hauner et al., 1998; Hotamisligil et al., 1995).

Obesity is frequently linked to insulin resistance and type-II diabetes.TNF-α blocked insulin-mediated glucose uptake in cultured cells (Hotamisligil et al., 1994a). TNF-α downregulated Glut4 mRNA levels in cultured adipocytes and myocytes (Stephens et al., 1997). It also reduced the catalytic activity of the insulin receptor. This effect was mediated through its ability in reducing the insulin-stimulated insulin receptor autophosphorylation and IRS-1 phosphorylation (Hotamisligil et al., 1994b). Neutralization of TNF-α with intravenous administration of TNFR-IgG in Zucker *fa/fa* rats dramatically improved insulin sensitivity and increased tyrosine phosphorylation of the insulin receptors and IRS-1 (Hotamisligil et al., 1994a, 1994b). In an *in vitro* study, TNF-α was shown to induce sustained suppression of cytokine signalling protein-3 (SOCS-3) production. This protein could decrease insulin-induced IRS-1 tyrosine phosphorylation and its association with the regulatory subunit, p85, of PI-3K. Therefore,

it was suggested that SOCS-3 might be a mediator in the insulin resistance developed with TNF-α treatment (Grimble, 2002).

Leptin has also been found to share sequence and structural homology with cytokines in the interleukin-6 (IL-6) family and their receptors. It is not surprising that it may also be involved in immunoactivity related to TNF-α. It has been known that high concentrations of leptin are seen in obese and diabetic patient. Many studies indicated that TNF-α might stimulate leptin secretion in adipocytes. TNF-α increased leptin secretion in a dose-dependent manner in primary cultures of epididymal adipocytes. An increase in steady-state leptin gene expression was observed following exposure of TNF-α. The mechanism how TNF-α affects leptin production is still unclear. TNF-α receptors may also mediate inflammatory hyperleptinemia by initiating a diverse signalling pathway after ligand binding. Studies have shown that p55 TNFR was essential for TNF-α to stimulate leptin secretion (Finck et al., 2000; Finck et al., 2002; Moller, 2000; Sethi et al., 1999). The links between increased levels of TNF-α and hyperleptinemia in obesity needs further clarification.

TNF-α can affect glucose, protein and lipid metabolism. It has been reported that prolonged infusion of TNF-α impaired insulin-mediated whole body glucose disposal and insulin-stimulated suppression of hepatic glucose output in rats (Moller, 2000). Downregulation of Glut4 mRNA expression and decrease in kinase activity of insulin receptor by TNF-α also impaired glucose homeostasis (Stephens et al., 1997). TNF-α could stimulate lipolysis by inhibiting the action of lipoprotein lipase, an enzyme responsible for the hydrolysis of lipoprotein triglycerides and for the entry of fatty acids into adipocytes. TNF-α also increased the activity of hormone-sensitive lipase, a rate-

limiting enzyme of lipolytic pathways (Argiles et al., 1997). These actions resulted in the elevation of serum lipid levels, such as triglyceride and free fatty acid (FFA) and a decrease in HDL cholesterol (Grimble, 2000; Ruan et al., 2003). Increased levels of FFA have been demonstrated to reduce glucose uptake and metabolism in rat heart and diaphragm muscles, and to inhibit insulin signalling and glycogen synthesis in muscle. Increased FFA oxidation also elevated the ratios of mitochondrial acetyl-CoA: CoA and NADH: NAD^+, resulting in impaired insulin-mediated glucose utilization. Therefore, TNF-α may influence the progress of insulin resistance by changing FFA levels (Ruan et al., 2003; Sethi et al., 1999)

1.5 Olanzapine-induced weight gain and cytokines

Olanzapine has been reported to induce dramatic weight gain in some patients, accompanied with elevated levels of glucose, lipid, insulin and leptin. As the hormones involved in the regulation of feeding behaviour and nutrient metabolism, the abnormalities of insulin and leptin will exert direct effects on glucose and lipid metabolism. These abnormalities may give rise to a development of insulin-resistance, resulting in drug-induced diabetes and increase the risk of cardiovascular disease.

Several clinical reports have shown that blood levels of TNF-α and/or TNFR are increased in antipsychotic-treated patients. Clozapine, which can cause obvious weight gain in patients, induces a significant increase in plasma levels of TNF-α, p55 TNFR and p75 TNFR after several weeks treatment (Pollmacher et al., 1996). Olanzapine, a structural analogue of clozapine, has also been reported to increase plasma level of TNFR in treated patients (Schuld et al., 2000). Atypical antipsychotic drugs can induce obesity

and increase the risk of developing type II diabetes in patients. The role of TNF-α needs to be evaluated in olanzapine-induced weight gain.

Several recent studies also showed that olanzapine increase feeding behaviour in animals (Arjona et al., 2004; Lee et al., 2002; Pouzet et al., 2003). A report attempted to analyze the relationship between the change of neurotransmitters and food intake. The neurotransmitters include dopamine, serotonin and their metabolites (Arjona et al., 2004). However, the mechanism of olanzapine-induced weight gain and the change of metabolism associated with weight gain are still unclear.

1.6 Hypothesis

It is hypothesized that increased level of TNF-α induced by olanzapine may be involved in the insulin and leptin resistance. In the brain insulin and leptin resistance may influence the feeding behaviour related to weight gain. In peripheral tissue, insulin and leptin resistance would lead to glucose and lipid abnormality as well as affecting adipocyte differentiation.

1.7 Objectives

The objectives of this study are to investigate the effects of olanzapine on feeding behaviour and to assess the potential role of insulin, leptin and TNF-α activities in olanzapine-induced weight gain and to examine any alteration of adipocytes in an animal model. The study is aimed to contribute to our understanding of the roles of cytokines in olanzapine-induced weight gain and to develop strategies to alleviate such unwanted side effects.

2. Materials and methods

2.1 Animal treatment

Female Wistar rats (200 - 250g, Charles River Canada, Montréal, Québec) were housed under a 12 h light-dark cycle at a temperature of 19-20^{o}C with free access to both food and water. All procedures involving animals were performed in accordance with the Canadian Council on Animal Care Guidelines and approved by University of Saskatchewan's Committee on Animal Care and Supply. After 1 week of acclimatization, rats were randomly divided into different groups. There were five animals in each group. Different concentrations of olanzapine were administrated *via* drinking water. Control group was treated with vehicle; other groups were treated with olanzapine at 0.003 mg/ml, 0.006 mg/ml, 0.01 mg/ml, 0.02 mg/ml, 0.03 mg/ml and 0.04 mg/ml. In another study, olanzapine (3 mg/kg) was given by oral administration. The average water intake by the rats was between 30 ml to 40 ml/day; therefore, the daily dosages of olanzapine treated rats were estimated between 0.3 mg/kg to 6 mg/kg. The food was ground rat chow mixed with canola oil. The weight ratio of rat chow to oil was 5:1 (5 ground chows: 1oil).

2.2 Drug

Olanzapine (Eli Lilly, Toronto, Canada) was dissolved in a small amount of 2% acetic acid and then was brought up to volume with distilled water, and neutralized with 10 M NaOH (pH = 6.0-7.0). Olanzapine tablets were also used. Inactive ingredients are carnuba wax, colour mixture white, hydroxypropyl cellulose, lactose and other inactive

component. For preparation of olanzapine solution, the tablets were dissolved in 2% acetic acid and filtered through 0.2 μm HT Tuffryn® membrane syringe filter. The filtered solution was diluted to the desired concentrations. The purity of olanzapine was determined by HPLC. It was not necessary to add an antioxidant to preserve olanzapine stability in samples as it was found to be stable under these conditions (Dusci et al., 2002). The HPLC system basically consisted of a solvent delivery pump, an automatic sample injector, a separation system, and an ultraviolet detector operated at 270 nm (figure.2.2.1). The separation system is consisted of a reverse phase C_{18} column, and a mobile phase of 14% acetonitrile in phosphate buffer (table 2.2.1), which was pumped at a flow-rate of 1 ml/min. Standard solution of olanzapine was prepared at a concentration of 0.03 mg/ml in water. 50 μl of olanzapine standard or olanzapine tablet solution was injected into the column. The stability of olanzapine upon storage was also assessed by the same method. Olanzapine solutions kept for 1 day at 4^{o}C, or 3 and 7 days at room temperature were tested.

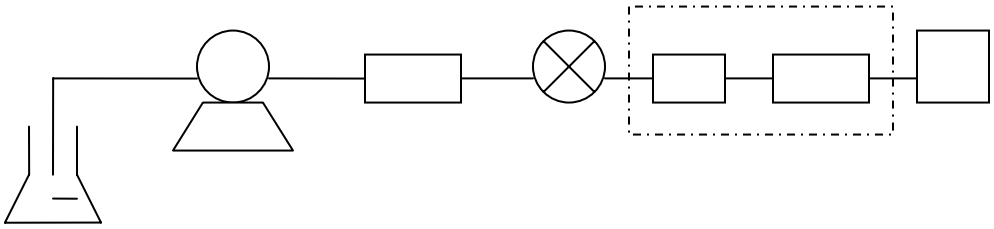


Figure 2.2.1 HPLC system. (1) mobile phase, (2) pump, (3) pre-column, (4) injector, (5) analytical column, (6) detector

Table 2.2.1. Preparation of 14% acetonitrile

Chemical compound	Final concentration	Amount in 1L
acetonitrile	14%	140 ml
H_3PO_4	0.25%	2.15ml
triethylamine	0.05%	0.43ml

2.3 Food intake, water intake and body weight measurement

The rat was housed individually for feeding measurement. Individual food and water consumption were assessed and recorded once a week. Food was provided in a bowl in each cage. The body weight of individual animals was assessed and recorded once a week.

2.4 Sample collection

2.4.1 Rat plasma

After fasting 12 hours, blood was collected from tail vein of the rats. 2% xylocaine gel was applied to the rat's tail for skin anaesthesia. The blood was collected by knife cut. Heparin was used as the anticoagulant. The blood sample was centrifuged at 2,000 g, at 4^{o}C for 10 minutes. The supernatant was collected as rat plasma. All samples were stored at –20^{o}C for further study.

2.4.2 Adipose tissue

Rats were anesthetised by an intraperitoneal injection of chloral hydrate at 40 mg/kg. The abdomen was rinsed with ethanol, and the skin was widely incised. The

subcutaneous adipose pad was collected, weighed and frozen on dry ice quickly. The abdomen was then opened and the abdominal adipose tissue was also collected, weighed and frozen in dry ice. All the samples were stored at –70^{o}C until further analysis.

2.5 Histochemistry

2.5.1 Hematoxylin stain

The slides were coated with 1% poly-L-lysine. The adipose tissue was cut into sections of 12-15 μm with a rotary microtome and dried at 37^{o}C. After being fixed in 4% paraformaldehyde for 1 minute, the section was stained using Mayer's hematoxylin for 5 minutes, and rinsed in the tap water following dehydration in a series of alcohols from 70%, 80%, 90%, and 95% to 100%. The slide was immersed in xylene for 3 minutes. The section was then mounted in xylene-based medium and observed under a light microscope.

2.5.2. Immunohistochemistry

The frozen subcutaneous adipose tissue was cut into 40 μm sections using a rotary microtome at -20ºC and then dried overnight at 37ºC. The sections were washed three times for 5 minutes each with PBS (pH 7.4) (table 2.5.1), then incubated in 3% hydrogen peroxide for 10 minutes to inactivate endogenous peroxidases. After washed three times using PBS for 5 minutes each, the sections were blocked by 5% rabbit serum for 10 minutes. Anti-collagen VI antibody (goat IgG) (diluted 1:500) was added to each section after removing the blocking serum. The sections were incubated overnight at 4°C. The sections were washed three times with PBS for 5 minutes each. Peroxidase-conjugated rabbit antigoat IgG (diluted 1:200) was added and incubated with sections for one hour.

After washing three times with PBS, DAB (3, 3'- Diaminobenzidine) peroxidase substrate solution was added to the sections. A brown color indicating the presence of the target epitope appeared within 5 minutes. The reaction was stopped by immersing the slides in tap water. Sections were dehydrated following sequential ethanol and xylene treatment. The procedure was initiated with a 3 minute wash with 70% ethanol, followed by 90%, 95%, 100% ethanol and finally with a xylene wash done twice. The slides were mounted with cover slips and were examined under a light microscope.

Table 2.5.1 Preparation of PBS for immunohistochemistry (Phosphate Buffered Saline, pH 7.4)

Chemical compound	Final concentration	Amount in 1L
NaCl	137.0 mM	8.0000 g/l
KCl	2.7 mM	0.2013 g/l
Na_2HPO_4	8.1 mM	1.1499 g/l
KH_2PO_4	1.5 mM	0.2041 g/l

2.6 Determination of triglyceride and glucose

2.6.1 Enzymatic assay for triglyceride in plasma

Triglycerides measurement involves enzymatic hydrolysis of triglycerides to glycerol and free fatty acids. The glycerol produced is then assessed following enzyme reactions by glycerol kinase (GK), glycerol -1- phosphate dehydrogenase (G-1-PDH) and diaphorase as indicated below:

$$\text{Triglycerides} \xrightarrow{\text{Lipoprotein lipase}} \text{Glycerol + Fatty acids}$$

$$\text{Glycerol + ATP} \xrightarrow{\text{GK}} \text{G-1-P + ADP}$$

$$\text{G-1-P + NAD} \xrightarrow{\text{G-1-PDH}} \text{DAP + NADH}$$

$$\text{NADH + INT} \xrightarrow{\text{Diaphorase}} \text{Formazan + NAD}$$

Glycerol is then phosphorylated utilizing ATP to form glycerol-1-phosphate (G-1-P) and ADP. The G-1-P is oxidized to DAP coupled with the reduction of NAD to NADH in the reaction catalyzed by G-1-P dehydrogenase (G-1-PDH). Finally, diaphorase catalyzes the reduction of 2-(p-iodophenyl)-3-p-nitrophenyl-5-phenyltetrazolium chloride (INT) to INTH (formazan) in the presence of NADH. Formazan, a coloured product, has an absorbance maximum at 500 nm. The intensity of the OD_{500} (optical density) is directly proportional to the triglyceride concentration of the samples. A liner standard curve was achieved (figure 2.6.1). The calculation of triglycerides concentration is shown as follows:

$$Triglycerides(mg/dl) = \frac{A_{test} - A_{blank}}{A_{calibrator} - A_{blank}} \times \text{concentration of calibrator}$$

Example:

A_{BLANK} = 0.054

A_{TEST} = 0.676

$A_{CALIBRATOR}$ = 0.442

$$Triglycerides(mg/dl) = \frac{0.676 - 0.054}{0.442 - 0.054} \times 250^{*} = 401$$

* Equivalent triglycerides concentration (mg/dL) of calibrator.

Blood samples were collected as described in “section 2.4.1”.

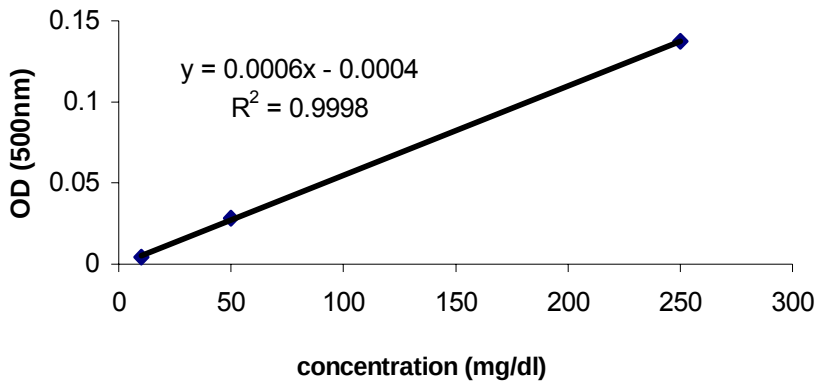


Figure 2.6.1 standard curve of triglyceride

2.6.2 Assay of blood glucose level

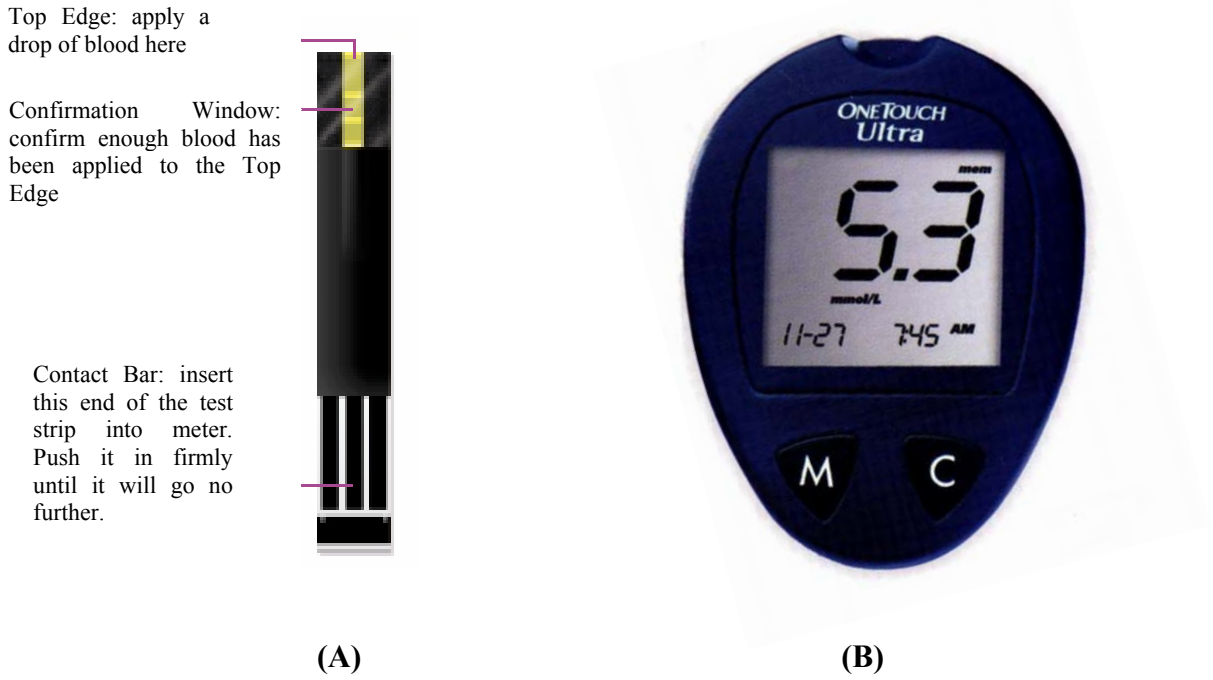
The levels of glucose in whole blood were assessed using ONETOUCH® Ultra test strips provide by Lifescan Company. The method is used as a home device for diabetic patients. It is a simple, quick, sensitive and accurate method with less pain, and needs less blood samples. The test principle is based on an enzymatic procedure and electrochemical measurement where

$$\text{glucose} + O_2 + H_2O \xrightarrow{\text{glucose oxidase}} \text{gluconate} + H_2O_2$$

A drop of blood (5 µl) is applied to the test strip. The glucose oxidase catalyzes the oxidation of the blood glucose to produce gluconate. During the reaction, electrons are transferred by an electrochemical mediator to the electrode surface. A small electrical current is produced and measured by the detector. The changes of the strength of the current represent the amount of glucose in blood.

Blood glucose levels were assessed after 12 hours fasting. Before collecting the blood sample, the test strip was inserted into the test port of the meter. The test strip then

touched and held the blood, where the blood met the narrow channel on the strip. The blood drop was held until the blood had completely filled the window before the meter began to count down. The blood glucose levels would appear on the display of the meter in 5 seconds.



(A) **(B)**

Figure 2.6.2 (A) ONETOUCH® Ultra test strip. (B) ONETOUCH® Ultra meter.

2.7 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA is a very sensitive method for quantitation assessment of specific proteins. ELISA was employed for the estimation of insulin, leptin and TNFα. The optimal conditions for ELISA were determined by the grid method. It is a method to test many antibody pair concentrations using only one plate (table 2.7.1).

For leptin standard curve, for example, high affinity 96 well microtitre plates (Maxisorb, Nunc) were coated with 100 µl/well phosphate buffered saline (PBS) (table 2.7.2) (pH7.4) containing 2 µg/ml goat anti-leptin IgG, and incubated overnight at room temperature. The plates were washed four times with PBS containing 0.05% Tween 20 (PBS-T) and then blocked for at least one hour at room temperature with 300 µl/well PBS containing 1% bovine serum albumin (BSA), 5% sucrose and 0.05% NaN_3. After four washes as above, 100 µl/ well standard leptin (62.5 pg/ml to 4 ng/ml), diluted in PBS-T containing 0.1% BSA (PBS-TB), or blood sample, was added into the plate and incubated at least 2 hours at room temperature. After washing the plate, 100 µl/well biotinylated goat anti-leptin IgG containing 100 ng/ml in PBS-TB was added to the plate and incubated for 2 hours at room temperature. Thereafter, 100 µl of extravidin-horse radish peroxidase, diluted in 1: 1000 in PBS-TB, was added per well to wash plates for 30 minutes. After washing, immunoreactivity was detected by adding 200 µl/well tetramethylbenzidine dihydrochloride (TMB) as peroxidase substrate for 30 minutes at room temperature. The reactions were stopped by the addition of 50 µl/well 1M H_2SO_4. The OD was determined at 450 nm by using a plate spectrophotometric plate reader and data were analysed by Softmax®pro software. Dose response standard curve of leptin between 62.5pg/ml and 4 ng/ml were obtained. Results were quite comparable from other labs (Hardie et al., 1996). The other standard curves, using recombinant insulin and TNFα, were conducted for each ELISA assay. Insulin, leptin and TNFα levels in blood samples were assessed from the standard curve on each plate utilizing a linear fit of data.

Table: 2.7.1 An example of grid experiment for leptin detection antibody detection assay

50 ng/ml detection	50 ng/ml detection	100 ng/ml detection	100 ng/ml detection	200 ng/ml detection	200 ng/ml detection
No leptin	No leptin	No leptin	No leptin	No leptin	No leptin
4 ng/ml leptin standard	4 ng/ml leptin standard	4 ng/ml leptin standard	4 ng/ml leptin standard	4 ng/ml leptin standard	4 ng/ml leptin standard

Table 2.7.2. Preparation of PBS for ELISA (Phosphate Buffered Saline, pH 7.4)

Chemical compound	Final concentration	Amount in 1L
KCl	2.7 mM	0.20 g
KH_2PO_4	1.5 mM	0.20 g
NaCl	137.0 mM	8.00 g
$Na_2HPO_4 \bullet 7H_2O$	8.06 mM	2.16 g

2.8 Adipocyte culture

2.8.1 3T3-L1 cell culture as a model for effect of olanzapine on triglyceride

3T3 –L1 cells are widely used for study of adipocyte differentiation due to their ability to differentiate into adipocytes, i.e. following treatment with agents including dexamethasone (Dex), isobutylmethylxanthine (IBMX) and insulin. In the present study, the 3T3-L1 cell line was used to exam the effect of olanzapine on triglyceride levels *in vitro*.

3T3-L1 cells, stored in liquid nitrogen, were thawed at 37°C, and then centrifuged at 1,200 rpm for 5 minutes. After discarding the supernatant, the cells were suspended by

1 ml preadipocyte culture medium containing 10% NBCS (table 2.8.1), and then transferred to 75 cm^2 flask with 20 ml medium for culture. These cells were maintained in an incubator at 37°C, 5% CO_2 for 3 days. Then the cells were washed with 0.9% saline solution and treated with 0.05% trypsin/0.02% EDTA solution for 30 seconds. After discarding the trypsin/EDTA solution, the cells were suspended with subculture medium containing 10% FBS. $5X10^5$ cells were then seeded in to the 24-well plate.

After confluence in 2 days, the medium was replaced by differentiation medium containing 10% FBS, insulin (2.5 μg/ml), Dex (0.25 μM) and IBMX (200 μM) (table 2.8.2). The cells were differentiated for 2 days. After differentiation, the cells were incubated in the maintaining medium containing 10% FBS and 2.5 μg/ml insulin for further study.

2.8.2 MTT test

MTT test is used to assay proliferation and viability of culture cells. As a tetrazolium salt, MTT can be cleaved to formazan by the "succinate-tetrazolium reductase" system, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. This system belongs to the respiratory chain of the mitochondria and is only active in metabolically intact cells.

Olanzapine at different concentrations, i.e. $1X10^{-6}$M, $1X10^{-5}$M, $1X10^{-4}$M, was added to medium. After 7-day's treatment in a 24-well plate, 200 μl MTT solution was added to each well and incubated at 37°C for 4 hours. Then 400 μl acid-isoproponal (solvent) was added to each well and mixed thoroughly to dissolve the purple crystals. The plate was incubated overnight at 37°C. 100 μl solution was transferred to each well of a 96-well plate. The plate was read at 570 nm.

2.8.3 Determination of cellular triglyceride levels in 3T3-L1 cells

3T3-L1 cells were treated with olanzapine at different concentrations, i.e. $1x10^{-5}$ M, $5x10^{-5}$ M, and $1x10^{-4}$ M. After 6-day's treatment, the medium was discarded. The cells were suspended in PBS after washed by cold PBS. The cells were then sonicated. The cellular triglyceride levels were detected by the method described in section 2.6.1.

Table 2.8.1 3T3-L1 preadipocytes culture medium base (1 L)

Chemical compound	Amount in 1 L
DMEM	10 g
NBCS or FBS	100 ml
D-glucose	3.5 g
HEPES	3.9 g
Sodium bicarbonate	1.5 g
Sodium pyruvate	0.11 g

The pH of the solution is adjusted with HCl to pH 7.2. The solution is filtered with 0.2 μm filter.

Table 2.8.2 3T3-L1 differentiation medium (100 ml)

Chemical compound	Amount in 100 ml
DMEM base	88.2 ml
FBS	10 ml
Penicillin (100 U/ml) / Streptomycin (100 μg/ml)	1 ml
Insulin (500 μg/ml)	1.0 ml
IBMX (0.1 M)	0.2 ml
Dex (0.25 mM)	0.1 ml

2.9 Assessment of protein profile in adipose tissue

2.9.1 Protein extraction and determinations

The frozen adipose tissue was immersed in 50 mM Tris-HCl buffer (pH 7.4) containing 1 M NaCl and protease inhibitor (aprotinin 10 μg/ml, leupeptin 10 μg/ml, pepstatin A 1 μg/ml, PMSF 10 μM), and then homogenized by using a polytron homogenizer. After centrifugation at 15,000 *g*, 4 °C for 10 minutes, the supernatant was collected for protein assay.

Protein concentration was determined by the Bradford method using Bio-Rad protein assay kit. A series of concentrations of Bovine serum albumin at 0, 0.016, 0.031, 0.062, 0.125, 0.25, 0.5 mg/ml, were used as standards. The absorbance of the solution was read at 595 nm using spectrophotometric plate reader and data were analysed by Softmax®pro software. A linear BSA standard curve was constructed, from which the protein concentration of the test sample could be determined.

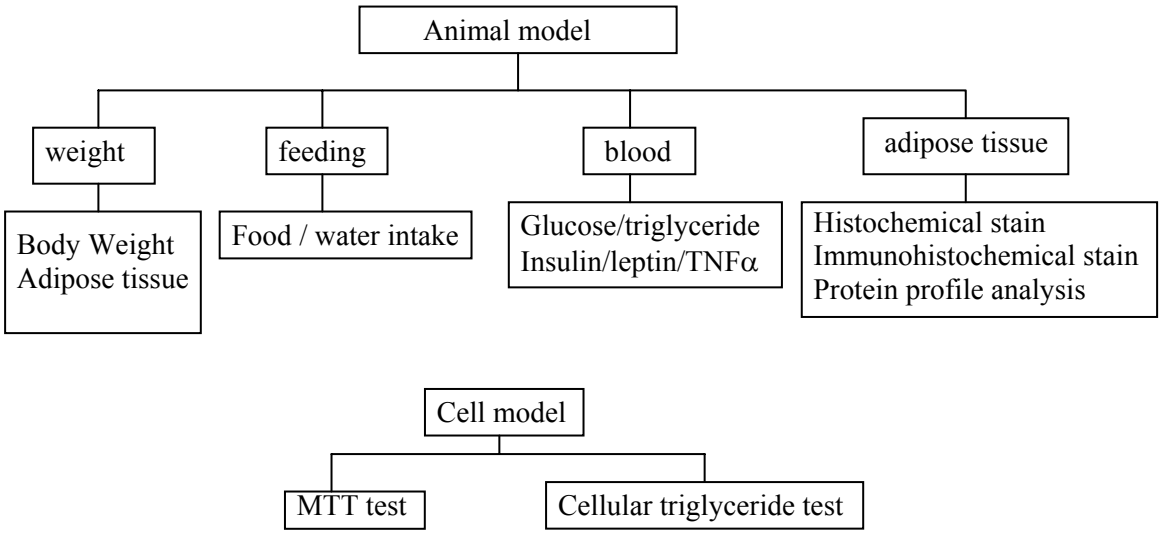
2.9.2 Assessment of protein profile

Equal amounts of total protein were electrophoresed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (15%). After electrophoresis, the protein profile on the gel was stained by a silver stain kit. A chromatic figure was obtained due to a redox reaction to reduce ionic silver to metallic silver occurred during the staining procedure. This method is 100 times more sensitive than Coomassie Blue and can detect protein at 0.1-1.0 ng levels.

2.10 Statistical analysis

Analysis of weekly weight gain, food and water intake by two-way analysis of variance (ANOVA) was performed using STAT program. Blood levels of glucose, triglyceride, insulin, leptin and TNF-α were analysed by one-way ANOVA using SPSS program. Individual comparisons between means were made using student's t- test. Correlation analysis was performed by Pearson's method using SPSS program.

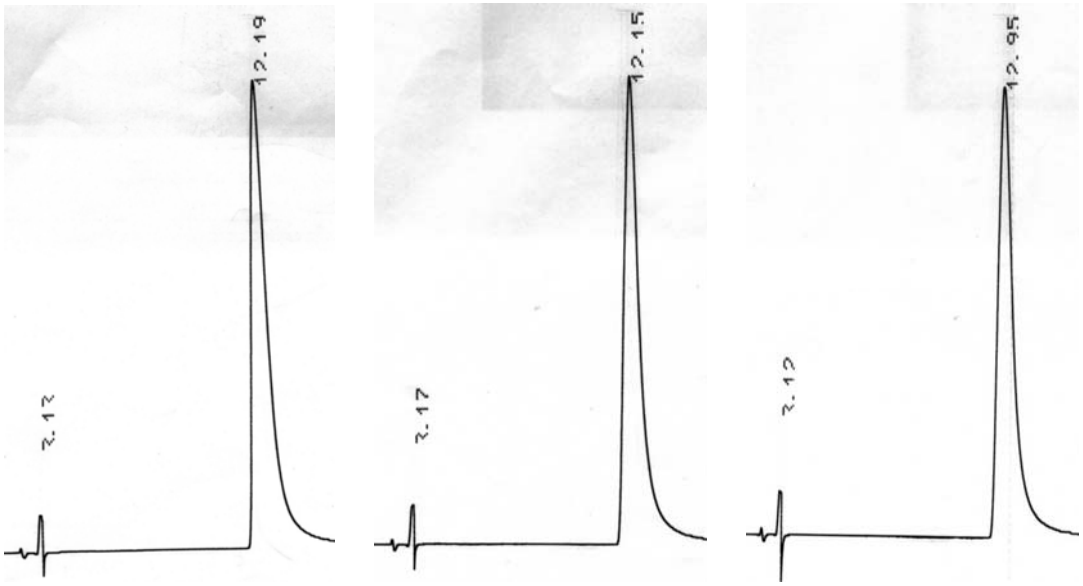
2.11 Research outline



3 Results

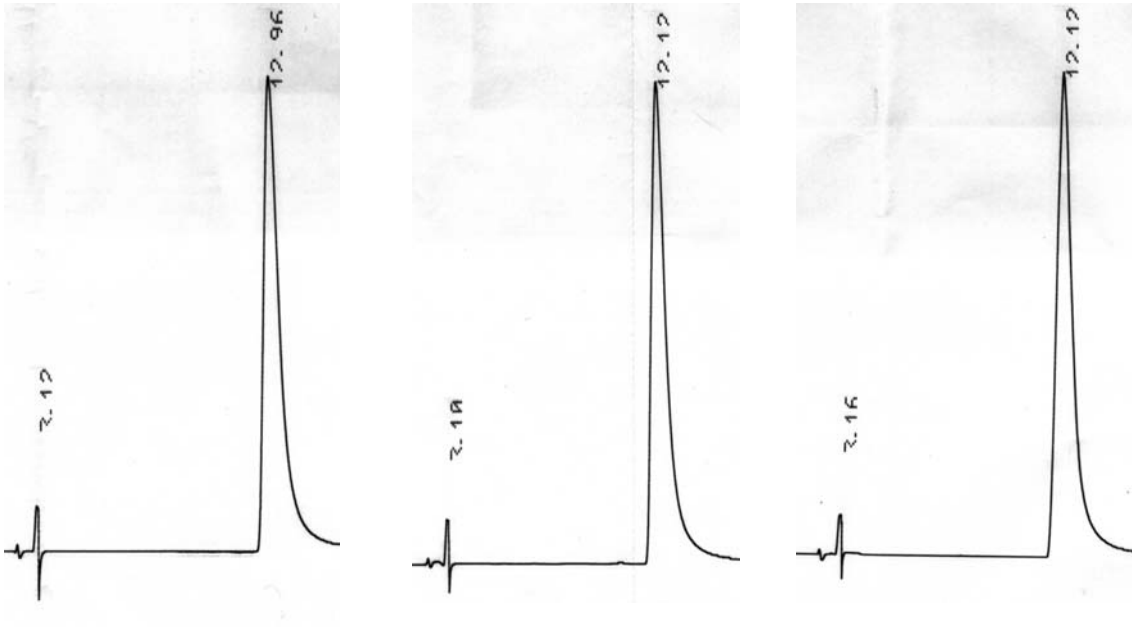
3.1 HPLC identification of olanzapine

Since in some studies olanzapine tablets were used, the content of olanzapine was estimated by HPLC. Pure olanzapine base was used as a reference standard. In the HPLC analysis, the time required for a target compound to elute is called retention time. Retention time for olanzapine under experimental condition is 12.19 minutes. The qualitative estimation was based on the peak surface area (or height). Figure 3.1.1 shows the chromatogram of olanzapine standards, diluted as 0.03 mg/ml, and olanzapine solutions from tablets containing olanzapine at 10 mg or 15 mg. Figure 3.1.2 shows the chromatogram of stability of olanzapine storage solution at 1, 3 and 7 days, respectively. The retention time of olanzapine is consistent with that of the pure compound. The content of olanzapine is exactly as it indicates for the tablet. Stock solutions prepared from olanzapine tablets are completely accurate as the concentrations indicated in the text.



(A) **(B)** **(C)**

Figure 3.1.1 HPLC chromatogram of Olanzapine. (A) pure olanzapine base (0.03 mg/ml). (B) olanzapine solution (0.03 mg/ml) (prepared from tablet containing 10 mg/tablet). (C) olanzapine solution (0.03 mg/ml) (prepared from tablet containing 15 mg/tablet). Volume for injection = 50 µl.



(A) **(B)** **(C)**

Figure 3.1.2 HPLC chromatogram of stability of olanzapine. (A) olanzapine solution (0.03 mg/ml) (storage for 1 day). (B) olanzapine solution (0.03 mg/ml) (storage for 3 days). (C) olanzapine solution (0.03 mg/ml) (storage for 7 days). Volume for injection = 50 µl.

3.2 Enzyme-Linked Immunosorbent Assay (ELISA)

The optimum leptin antibody concentration is determined by grid experiment. The result displayed in figure 3.2.1 shows that the optimal range of concentrations for detection of leptin is anti-leptin goat IgG at 2 ng/ml and biotinylated anti-leptin goat IgG at 100ng/ml. The ELISA performed under these conditions are capable of detecting pictogram levels of leptin with low background (OD < 0.2).

The standard curve obtained for leptin, insulin by ELISA is linear fit (Figure 3.2.2). Knowing the absorbance of the sample assayed and the equation for the line of the standard curve, the concentration of cytokines in a sample is calculated as follows:

$y = ax + b$

$x = (y-b) \div a$

where: y = absorbance of sample at 450 nm

x = sample cytokine concentration (ng/ml)

a = slope of standard curve

b = y – intercept of standard curve

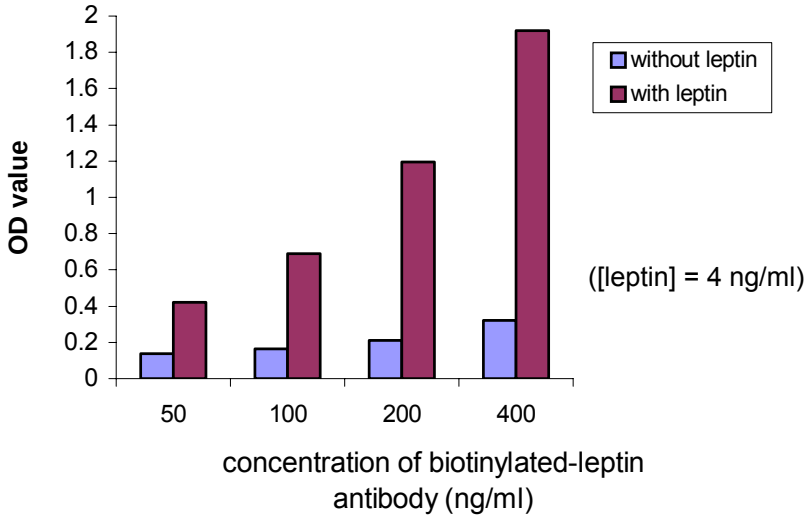
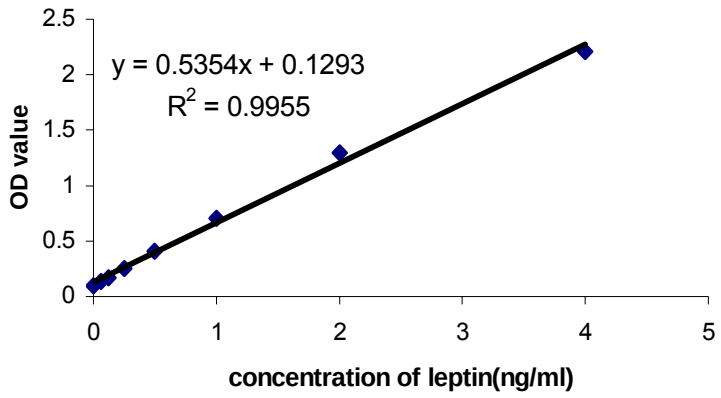
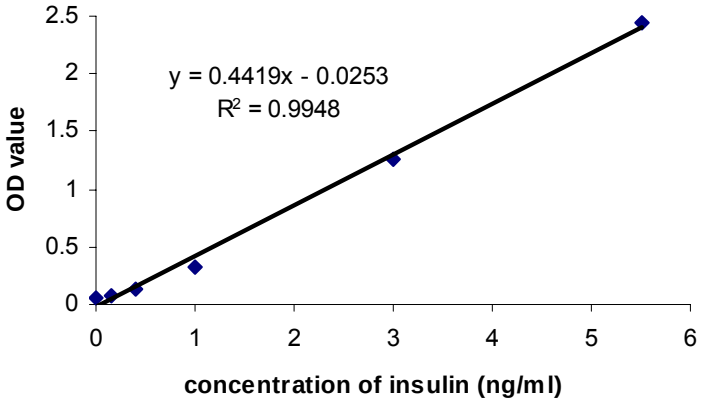


Figure 3.2.1 Optimizing leptin antibody concentrations by grid experiment.





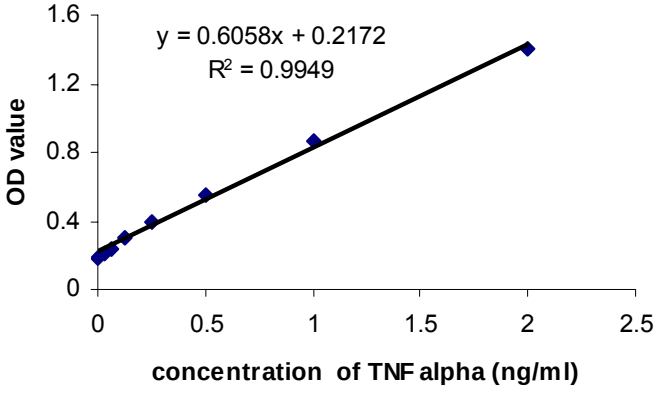


Figure 3.2.2 Standard curve of leptin, insulin and TNFα by ELISA

3.3 Effects of olanzapine on cumulative body weight gain

As can be seen in figure 3.3.1, olanzapine induced significant increases in cumulative weight gain in female rats over the 4-week treatment period relative to control group ($p<0.05$). In week 1 and 2, a rapid growth was seen in the group treated with olanzapine at 0.03 mg/ml drinking water. Cumulative body weight gain remained constant over the duration of the test period. A dose–related increase in cumulative body weight gain was observed. The group treated with 0.03 mg/ml experienced marked weight gain just after 1-week treatment and was constant for four weeks. However, the group treated with lower concentration of olanzapine (0.01 mg/ml) began to gain weight significantly only after the second week of treatment.

In the subsequent experiment, different concentrations of olanzapine (0.02 - 0.04 mg/ml) were used. Twice/per week fasting and weekly blood collection were conducted. Olanzapine did not yield a significant effect on weight gain. It seems that olanzapine failed to produce weight gain and this might be due to these relatively invasive treatments (fasting and venous blood collection) (Figure 3.3.2).

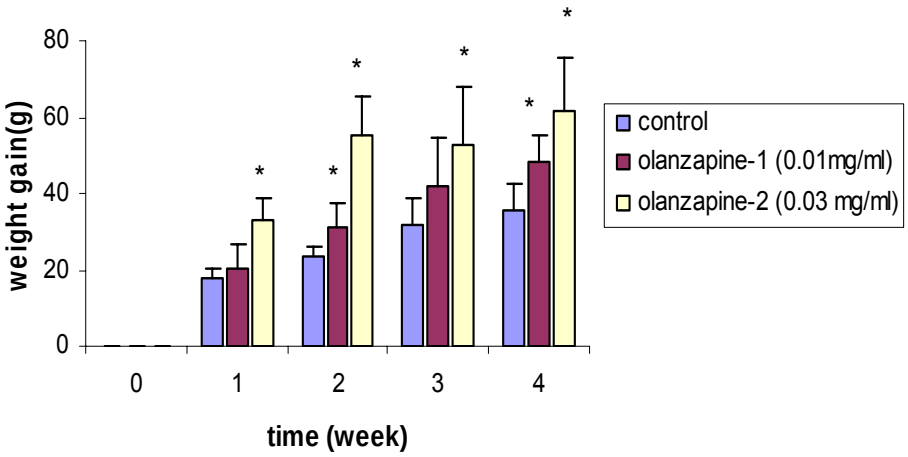


Figure3.3.1. Chronic effects of olanzapine administered *via* drinking water (0.01 - 0.03 mg/ml) on cumulative body weight gain in Wistar female rats. Values represent mean ± S.D.; n = 5 per group (* $p \leq 0.05$).

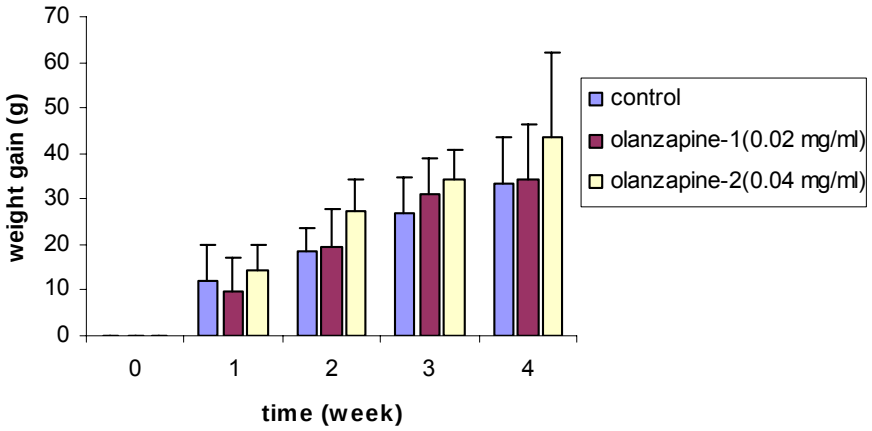


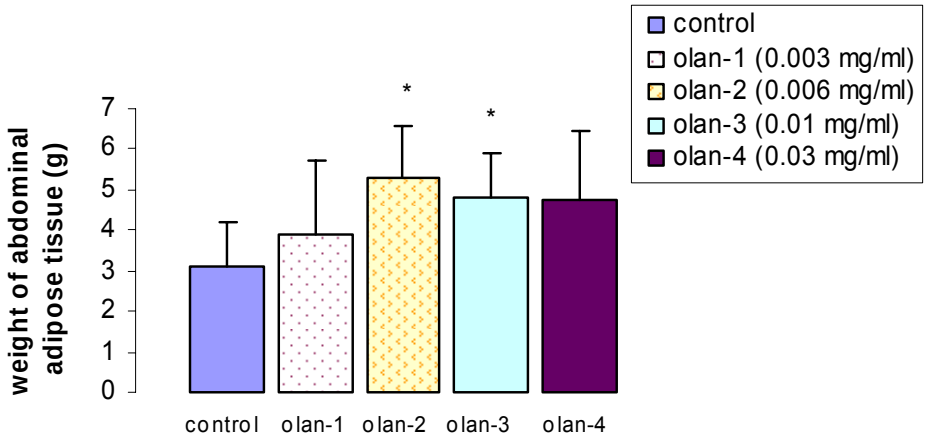
Figure3.3.2. Chronic effects of olanzapine administered *via* drinking water (0.02 - 0.04 mg/ml) on cumulative weight gain in Wistar female rats. Animals were subjected to repeated harsh treatment (i.e. fasting and collection of venous blood. Values represent mean ± S.D.; n = 5 per group

3.4 Effect of olanzapine on the weight of adipose tissues

The chronic effect of olanzapine on the total mass of subcutaneous and abdominal adipose tissue in female Wistar rats was also assessed. A significant increase in both tissues was detected in animals treated with olanzapine (0.006 mg/ml and 0.01 mg/ml) (Figure 3.4.1). A large within-group variance was also observed in olanzapine-treated group, especially in the group treated with dose of 0.03 mg/ml, which renders the increase of fat tissue masses not significant.

Correlation analyses were performed on the weight gain vs. the masses of adipose tissue. The results revealed that there was a significant positive correlation between weight gain and the weight of adipose tissue from two distinct regions ($p < 0.01$) (Figure 3.4.2).

(A)



(B)

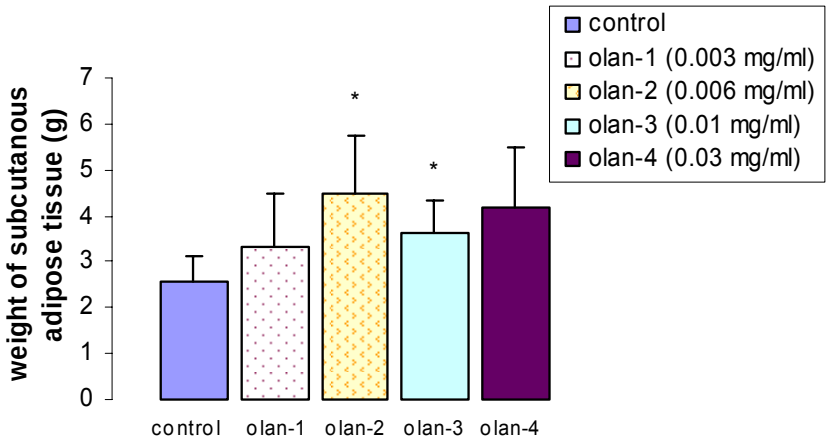
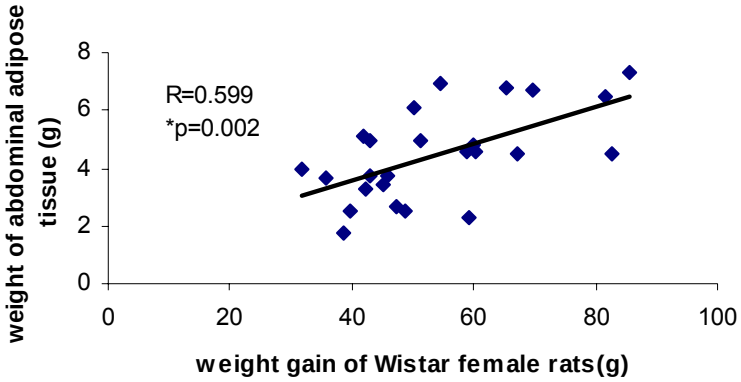


Figure 3.4.1 Effect of olanzapine administered *via* drinking water (0.003-0.03 mg/ml) on the mass of (A) abdominal adipose tissues, and (B) subcutaneous adipose tissue in Wistar female rats after 4-week treatment. Values represent mean ± S.D.; n = 5 per group (* $p \leq 0.05$)

(A)



(B)

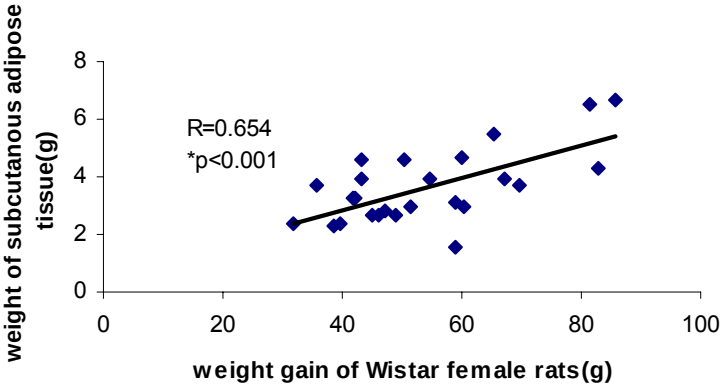


Figure 3.4.2 Scattergram of the relationship between weight gain and total mass of (A) abdominal adipose tissue, and (B) subcutaneous adipose tissue of rats after four-week olanzapine-treatment.

3.5 Effect of Olanzapine on food intake and water intake

The effect of olanzapine on food and water intake was also measured, as shown in figure 3.5.1. Olanzapine induced a significant increase in food intake, but this was observed only after one-week treatment. During the following 4-week study olanzapine did not significantly affect food intake.

The effect of olanzapine on water intake is shown in figure 3.5.2. The drug appeared to increase water consumption after one-week treatment and maintained its effect up to third week. However, significant increases were not obtained at all time points or concentrations of olanzapine. Olanzapine at 0.006 mg/ml induced a marked increase in water consumption in week 3 ($p < 0.05$), and at 0.01 mg/ml increased water intake significantly in week 1 ($p < 0.05$). No effect of olanzapine at 0.003 mg/ml and 0.03 mg/ml were observed on water intake in female rats compared to the control group. The large individual variation makes it difficult to find any significant effect.

The following analysis is to determine whether food intake was directly related to weight gain. In this analysis, all treated animals were pooled and divided into high and low weight gain group based on the mean value of their weight gain (54.5 g), without considering the concentration of olanzapine. Olanzapine significantly increased food consumption in rats with high weight gain also only in week 1 ($p < 0.05$) (Figure 3.5.3). This effect disappeared after two-week treatment. However, olanzapine did appear to increase feed efficiency in drug-treated rats with high weight gain. Feed efficiency is defined as weight gain (g)/food intake (g). Interestingly, a significant lower feed efficiency was observed in low weight gain group in week 1, 2 and 4 ($p < 0.05$) (Figure 3.5.4).

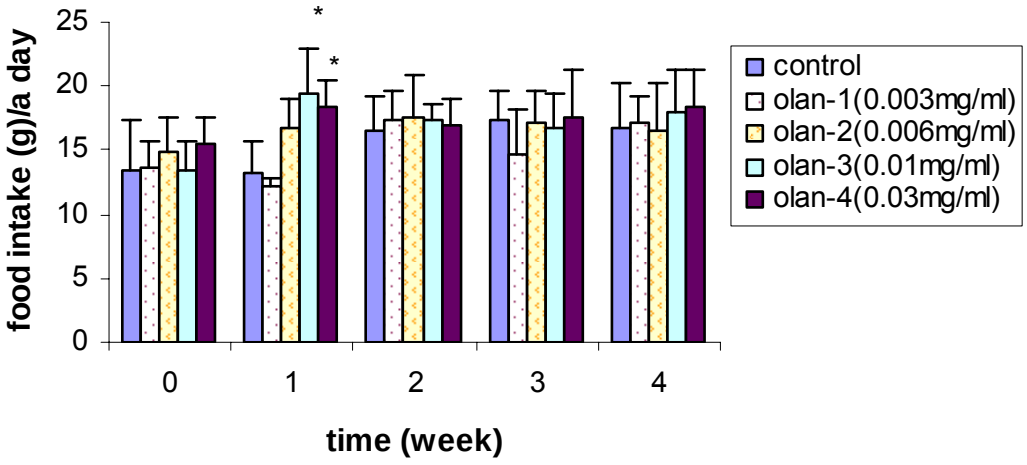


Figure 3.5.1 Effect of olanzapine administered *via* drinking water (0.003-0.03 mg/ml) on food intake in Wistar female rats. Values represent mean ± S.D.; n = 5 per group (* $p \leq 0.05$)

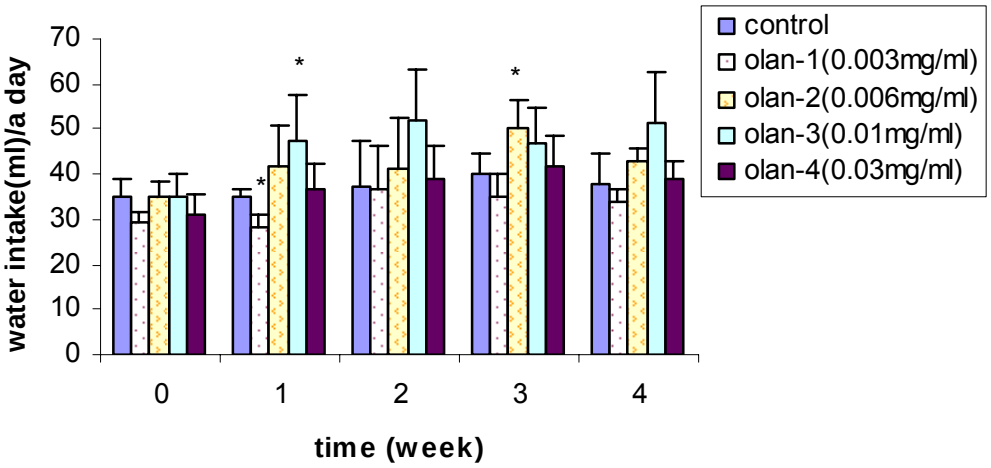


Figure 3.5.2 Effect of olanzapine administered *via* drinking water (0.003-0.03 mg/ml) on water intake in Wistar female rats. Values represent mean ± S.D.; n = 5 per group (* $p \leq 0.05$)

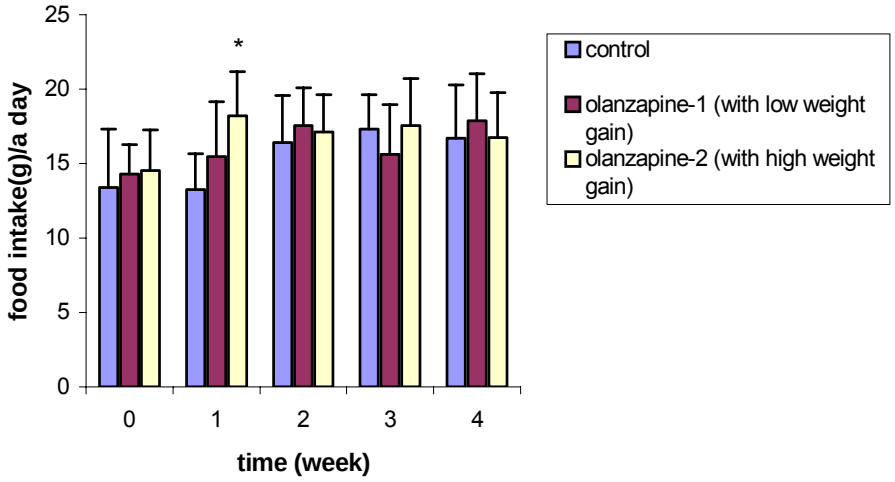


Figure 3.5.3 Effect of olanzapine administered *via* drinking water (0.003 mg/ml to 0.03mg/ml) for 4 weeks on food intake in relationship to weight gain in Wistar female rats. Olanzapine-treated animals were regrouped based on difference of weight gain (Low weight gain < 54.5g, high weight gain > 54.5g). Values represent mean ± S.D.; n = 5; 11; 9 (* $p \leq 0.05$)

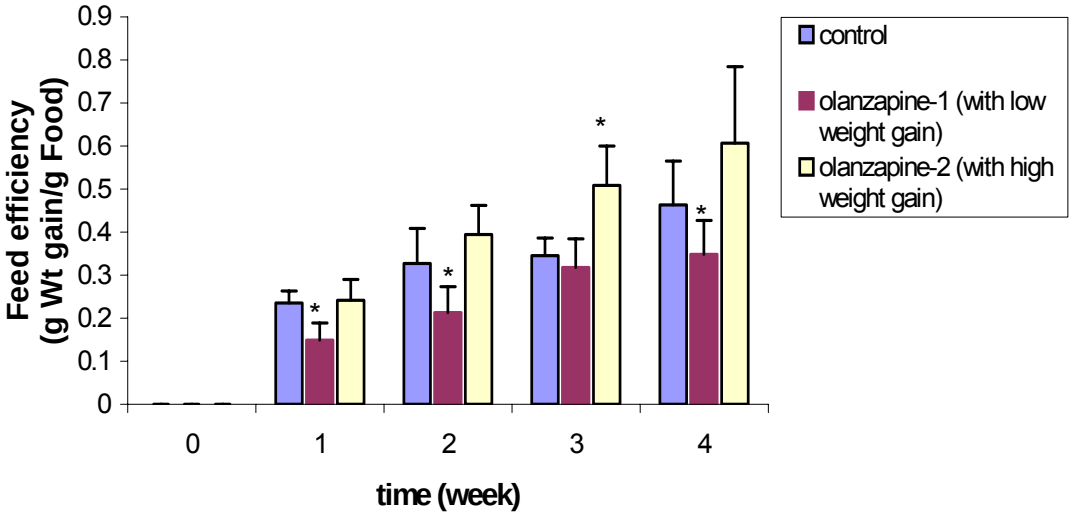


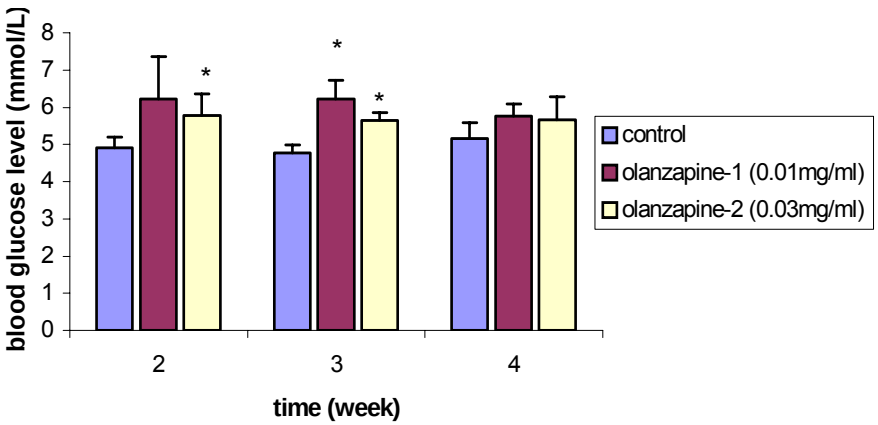
Figure 3.5.4 Effect of olanzapine on feed efficiency in relationship to weight gain in Wistar female rats. Olanzapine-treated animals were regrouped based on difference of weight gain (Low weight gain < 54.5g, high weight gain > 54.5g). Values represent mean±S.D.; N = 5; 11; 9 (* $p \leq 0.05$)

3.6 Effect of olanzapine on blood glucose and plasma triglyceride levels

Blood glucose levels are increased in olanzapine-treated animals. As can be seen in figure 3.6.1- (A), a significant increase in glucose levels was detected in rats treated with 0.01 mg/ml of olanzapine during week 3, and in rats treated with 0.03 mg/ml during week 2 and 3. A follow-up experiment shows that glucose levels in animals treated with different concentrations of olanzapine were not different from that of control animals in week 4 (Figure 3.6.1-(B)).

Plasma triglyceride levels were unchanged in these rats following olanzapine treatment for 4 weeks (Figure 3.6.2).

(A)



(B)

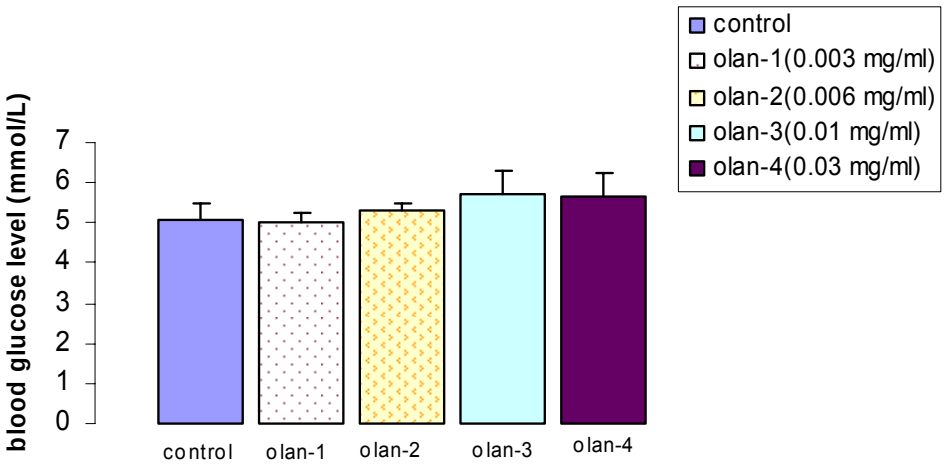


Figure 3.6.1 Effect of olanzapine on blood glucose levels in Wistar female rats. Olanzapine was administered *via* drinking water after 4 weeks. Values represent mean ± S.D.; n = 5 per group (* $p \leq 0.05$)

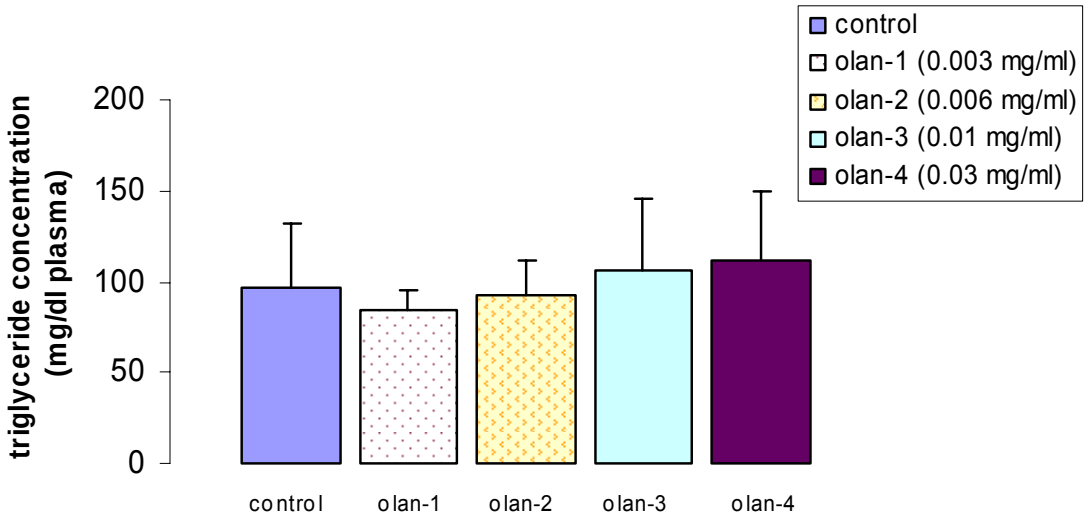


Figure 3.6.2 Effect of olanzapine on plasma triglyceride levels in Wistar female rats. Olanzapine was administered *via* drinking water for 4 weeks. Values represent mean ± S.D.; n = 5 per group.

3.7 Effect of olanzapine on viability and triglyceride content in cultured adipocytes

In order to assess whether olanzapine directly affect triglyceride level in adipocytes, an adipocyte cell culture, 3T3-L1cell line were employed. *In vivo*, olanzapine is extensively distributed throughout the body. 93% of olanzapine would be bound to plasma proteins over the concentration range of 7 to 1100 ng/ml. Based on the plasma concentrations, concentrations of olanzapine, 1×10^{-6} M to 1×10^{-4} M, were used in the present cell experiment.

At the concentrations of 1×10^{-6} M to 1×10^{-4} M, Olanzapine does not seem to affect the viability of 3T3-L1 adipocytes *in vitro* after 7-day's treatment (figure 3.7.1). Neither does it alter the cellular triglyceride levels of differentiated 3T3-L1 adipocytes after 6-day's treatment (figure 3.7.2).

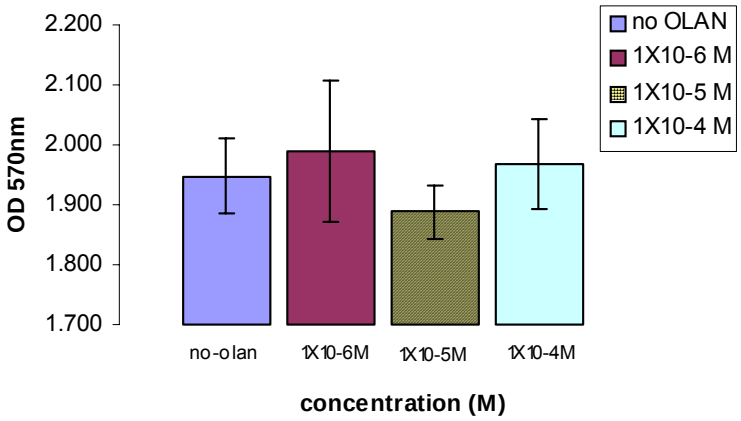


Figure 3.7.1 Effect of olanzapine on viability of 7-day's treated differentiated 3T3-L1 adipocytes by MTT test. Values represent mean±S.D. n = 4 wells per group.

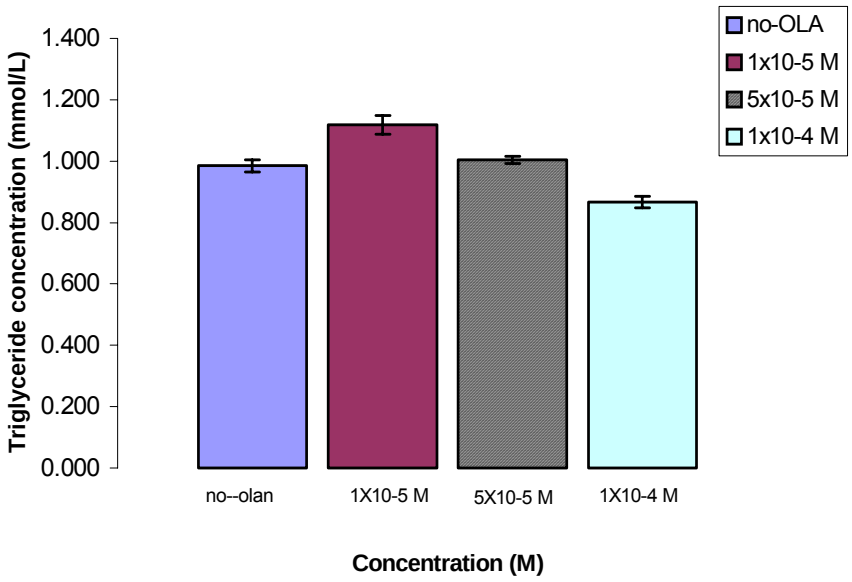


Figure 3.7.2 Effect of olanzapine on cellular triglyceride levels of 6-day's treated differentiated 3T3-L1 adipocytes. Values represent mean±S.D. n = 4 wells per group.

3.8 Effect of Olanzapine on plasma levels of leptin, insulin and TNFα

In Wistar female rats, olanzapine administered *via* drinking water induced a significant increase in plasma insulin levels after four-week treatment (Figure 3.8.1). However, olanzapine did not significantly affect levels of plasma leptin and TNF-α after four-week treatment. A very large individual variance in these animals was observed (Figure 3.8.2).

However, after regrouping the olanzapine-treated animals based on their weight gain at the end of the treatment, analysis revealed that plasma levels of insulin and leptin were significant correlated to the weight gain in the treated rats ($p < 0.05$, Figure 3.8.3).

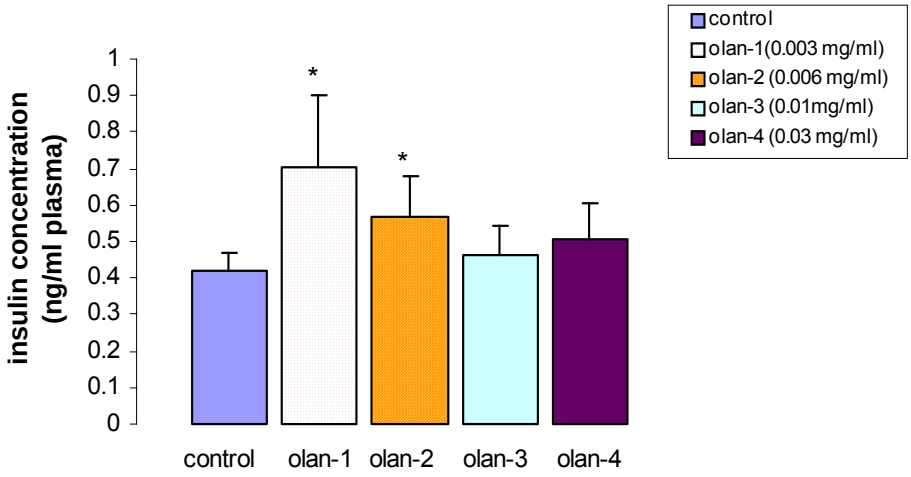
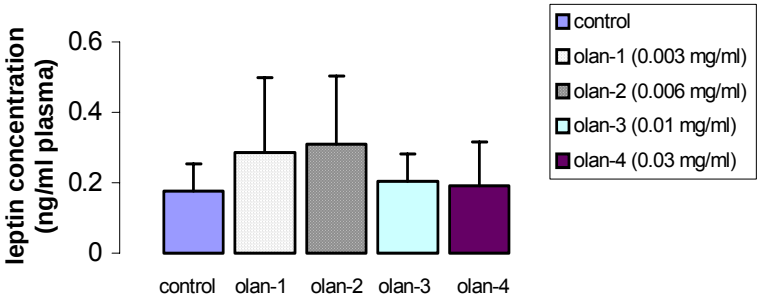


Figure 3.8.1 Effect of olanzapine administered *via* drinking water (0.003-0.03 mg/ml) on plasma insulin levels in Wistar female rats after four weeks of treatment. Values represent mean ± S.D.; n = 5 per group (* $p \leq 0.05$)

(A)



(B)

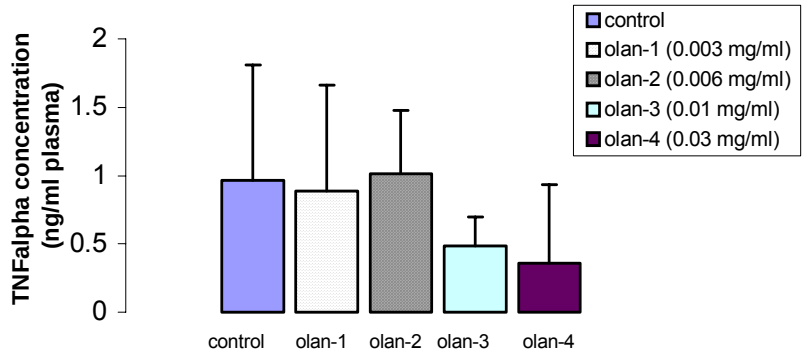
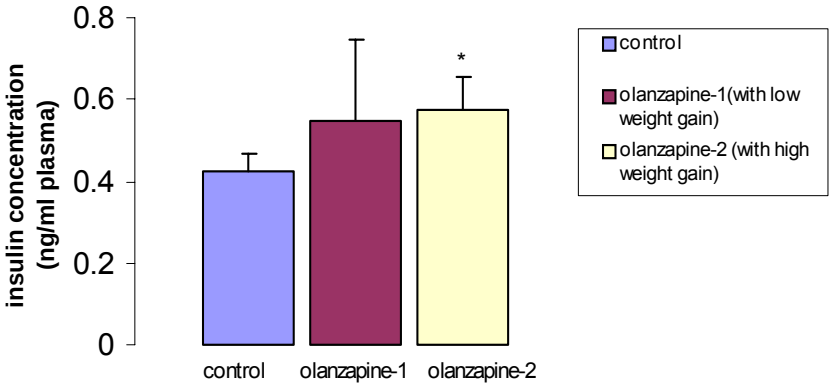


Figure 3.8.2 Effect of olanzapine administrated *via* drinking water (0.003-0.03 mg/ml) on plasma (A) leptin levels and (B) TNFα levels in Wistar female rats after four weeks of treatment. Values represent mean ± S.D.; n= 5 per group

(A)



(B)

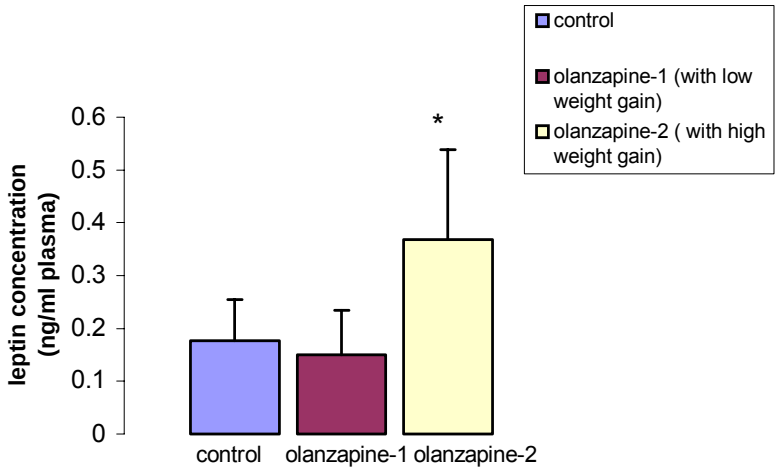


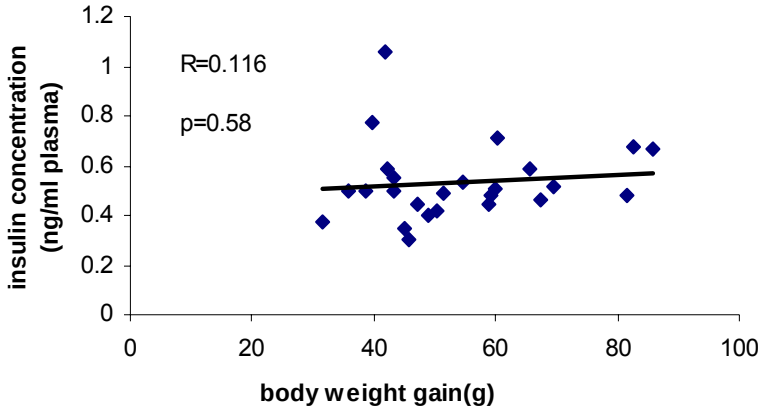
Figure 3.8.3 Effect of olanzapine on plasma levels of (A) insulin and (B) leptin in Wistar female rats. Olanzapine-treated animals were divided to two groups (Low weight gain < 54.5g, high weight gain > 54.5g). Values represent mean ± S.D.; n = 5; 11; 9 (* $p \leq 0.05$)

3.9 Correlation between plasma levels of insulin, leptin, weight gain and weight of adipose tissues

In order to reveal how plasma insulin and leptin are related to olanzapine-induced weight gain, correlation analyses were performed between the levels of insulin, leptin and weight gain after four weeks of treatment. The scatter gram is shown in figure 3.9.1 (A). The correlation (R = 0.116) observed between insulin levels and weight gain was not statistically significant (p = 0.58). However, a positive correlation was found between leptin level and weight gain (R= + 0.537, p=0.006) (Figure 3.9.1(B)).

Correlation analyses were also performed on the levels of insulin and leptin vs. the masses varies adipose tissues. Once again no correlation was found between insulin levels and the mass of subcutaneous and abdominal adipose tissue (p=0.487 and 0.375, respectively) (Figure 3.9.2). A marked positive correlation was observed between leptin levels and both adipose tissues (p = 0.006 and 0.003, respectively) (Figure 3.9.3).

(A)



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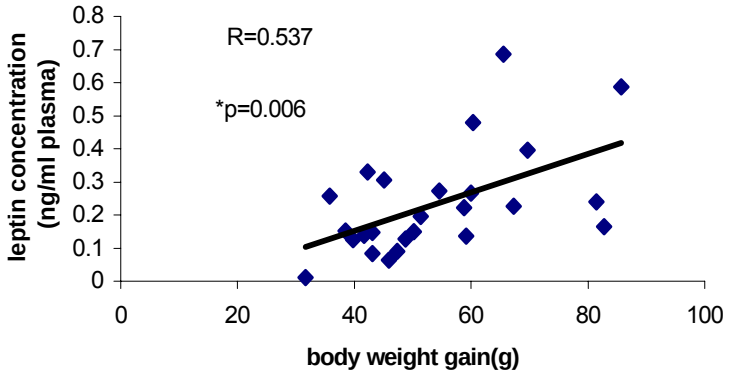
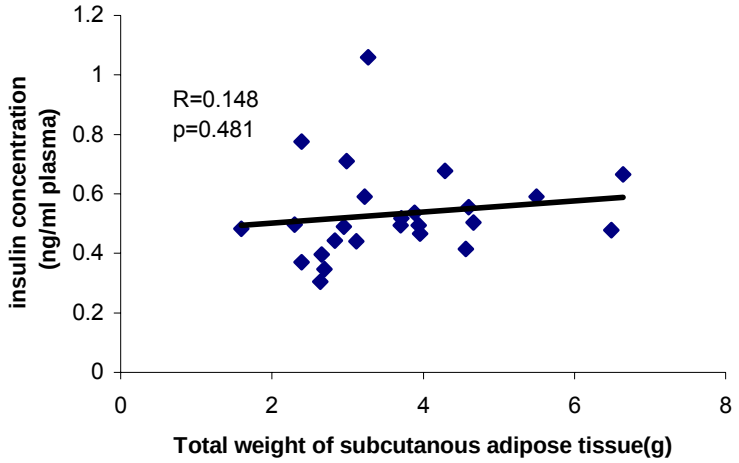


Figure 3.9.1 Scattergram of correlation between weight gain and plasma level of insulin and leptin in rats after four-week olanzapine treatment.

(A)



(B)

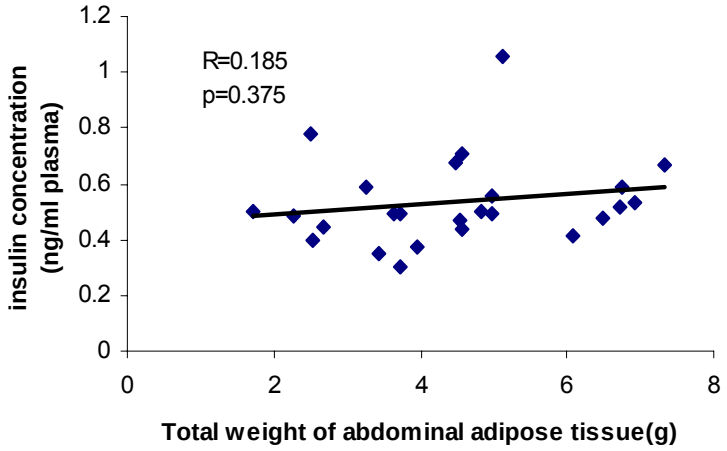
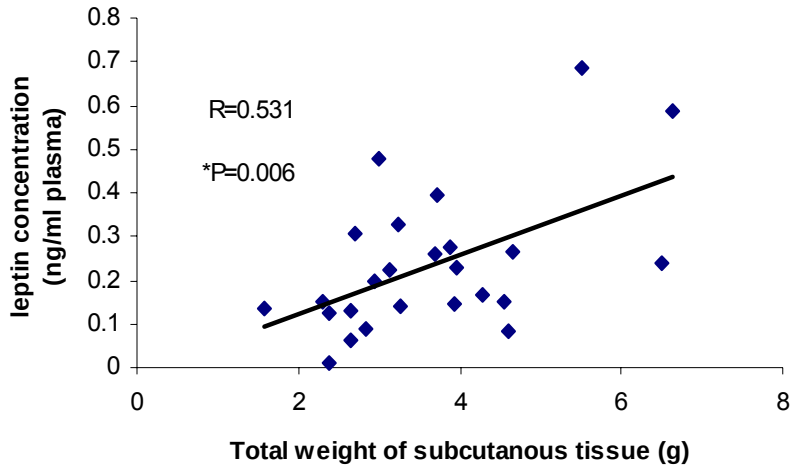


Figure 3.9.2 Scattergram of correlation between total masses of various adipose tissues and plasma levels of insulin of rats after four-week olanzapine treatment.

(A)



(B)

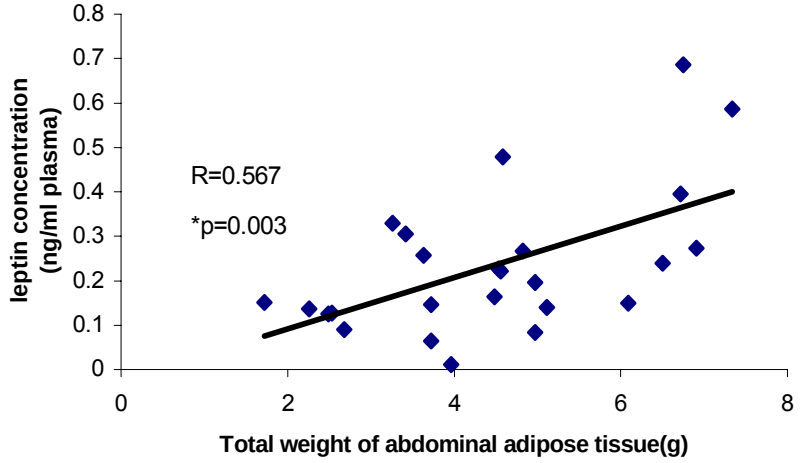


Figure 3.9.3 Scattergram of correlation between total masses of adipose tissues and plasma levels of leptin of rats after four-week olanzapine treatment.

3.10 Olanzapine alters some morphological appearance of subcutanous adipose tissue

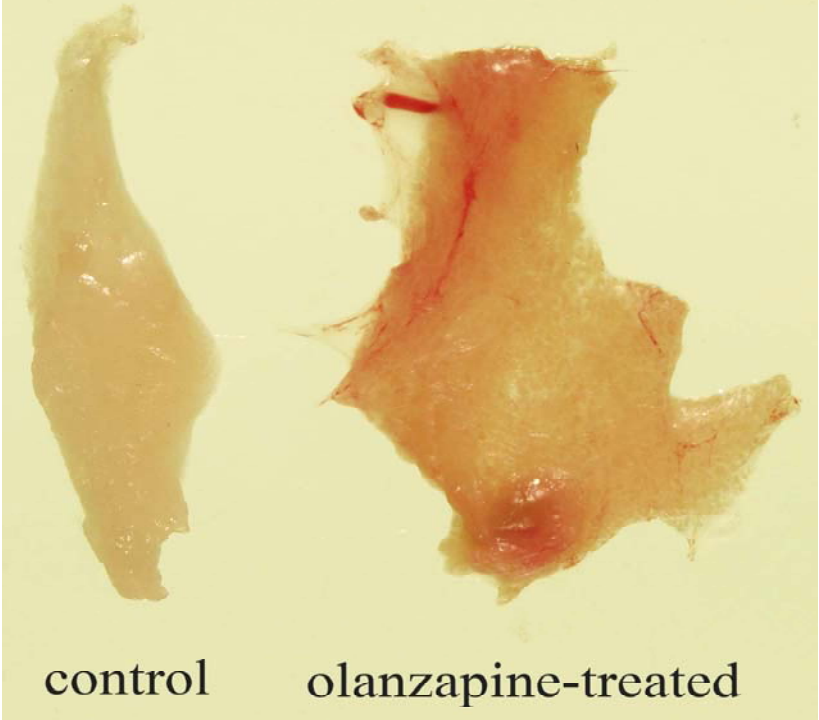
A consistent effect of olanzapine observed in this study was the dramatic morphological changes in the subcutanous adipose tissue. The adipose tissues from control animals were white, bright with puffy appearance. In contrast, the olanzapine-treated adipose tissues were yellowish to brownish, non-transparent with "fish egg-like" granule appearance (Figure 3.10.1). Apparent enlarged vessels in the adipose tissues of the olanzapine-treated animals were also observed.

Since quantification method for the morphological changes in fat adipose tissue is not available, the degree of changes were visually assessed and independently confirmed by other colleagues.

After 8-week treatment with olanzapine at concentration of 0.02 mg/ml and 0.04 mg/ml, the morphological change in subcutaneous adipose tissue was observed in all the treated animals (Table 3.10.1, 3.10.2). The changes were semi-quantified from "-" (representing white colour or puffy appearance) to "+" (sparsely or faint colorization and fish-egg appearance), "++" (intermediate of colorization and fish-egg appearance) to "++" (representing yellow colour or fish-egg appearance).

It is interesting to note that these morphological changes were not generally applied to all adipose tissues. Indeed, no morphological changes in abdominal adipose tissues in the same olanzapine-treated rats were observed.

(A)



(B)

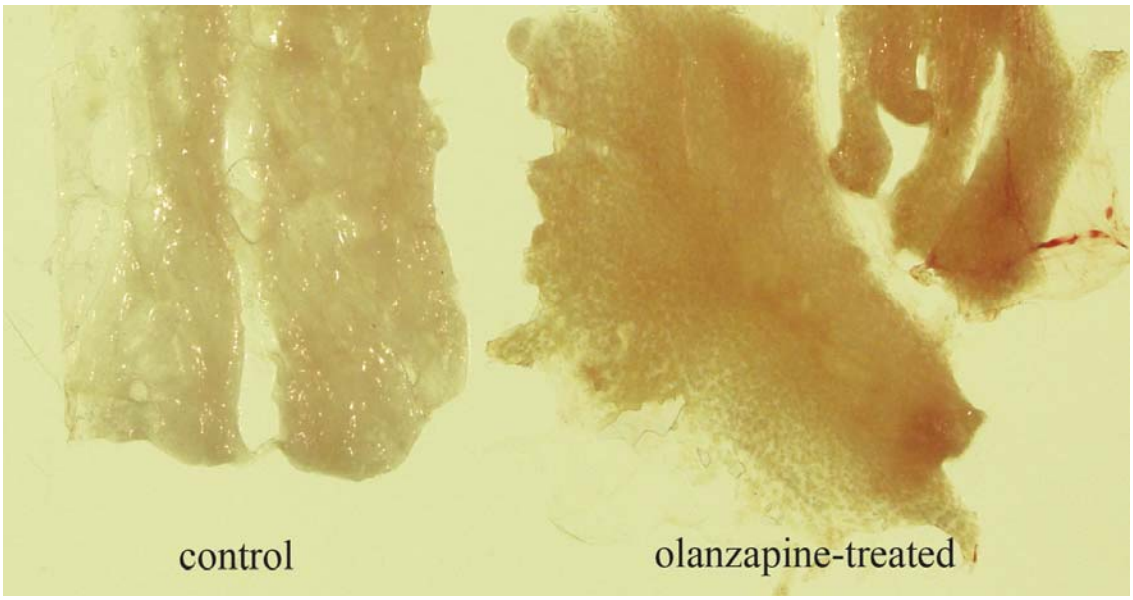


Figure 3.10.1 Effect of chronic treatment of olanzapine on morphological changes of subcutanous adipose tissues in Wistar female rats (A) gross morphological change; (B) magnification: 2X.

Table 3.10.1 Effect of olanzapine on the colour of subcutanous adipose tissues in Wistar female rats. Rats were treated with vehicle or olanzapine for 8 weeks. n = 5 per group. (white : -, yellow: +).

Group (n=5) No.	Control	Olanzapine-1 (0.02 mg/ml)	Olanzapine-2 (0.04 mg/ml)
1	–	+/++	++
2	–	+	+/++
3	–	+	+++
4	–	+/++	++
5	–	++	++

Table 3.10.2 Effect of olanzapine on the texture of subcutanous adipose tissues in Wistar female rats. Rats were treated with vehicle or olanzapine for 8 weeks. n = 5 per group. (puffy-appearance: -, fish egg-appearance: +).

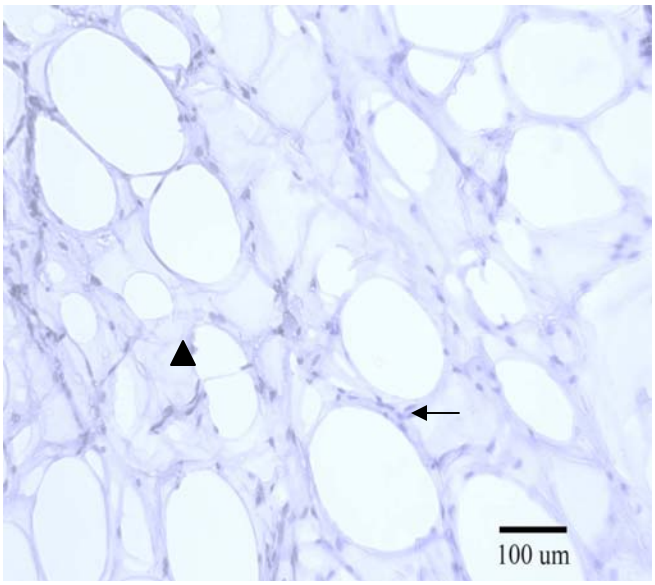
Group (n=5) No.	Control	Olanzapine-1 (0.02 mg/ml)	Olanzapine-2 (0.04 mg/ml)
1	–	+/++	++
2	–	++	++
3	–	+	+++
4	–	++	++
5	–	++	++

3.11 Histochemical examination of the adipose tissues

The histological examination using hematoxylin stain revealed the difference between adipose tissues obtained from control and olanzapine treated animals with olanzapine. As can be seen in figure 3.11.1, more nuclei and extracellular matrix structure were observed surrounding the adipocytes in olanzapine-treated animals.

It is known that hematoxylin is capable of staining collagen. Therefore, a specific immunohistochemical stain of collagen VI was employed to examine the adipose collagen. A strong positive stain was observed in olanzapine-treated adipocyte (Figure 3.11.2). It is clear that the collagen VI protein was increased in the extracellular matrix adipose tissues from olanzapine-treated rats.

(A)



(B)

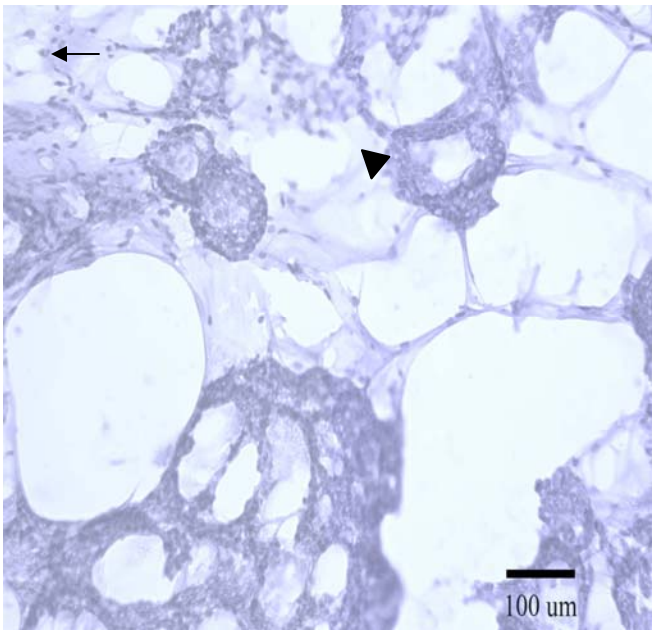
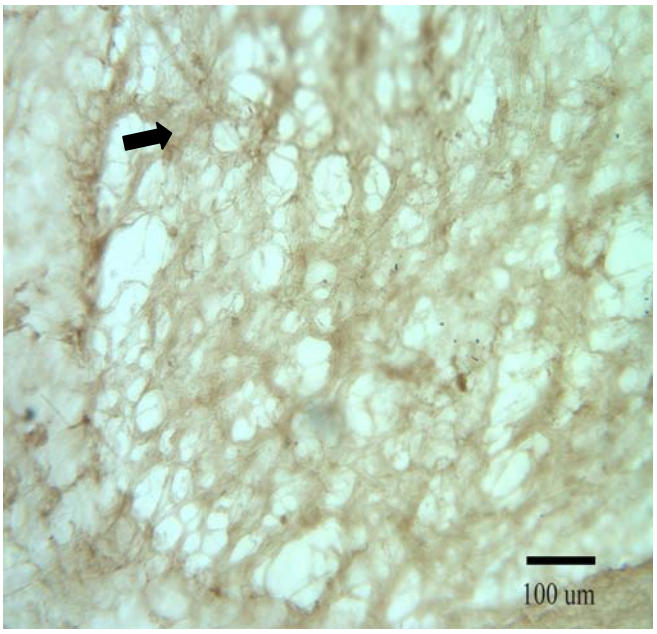


Figure 3.11.1 Histochemical examination of subcutanous adipose tissues in control and 4-week olanzapine-treated (0.03 mg/ml) Wistar female rats (↑: nucleus, ►: extracellular matrix). (A) hematoxylin-stained normal adipocytes from control animals; (B) hematoxylin-stained adipocytes from olanzapine-treated animals. Original magnification: 100X; scale bar:100 μm.

(A)



(B)

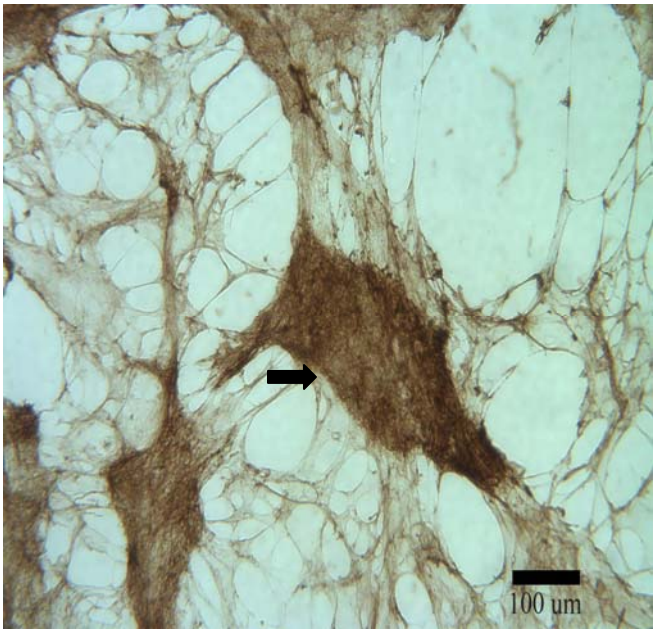


Figure 3.11.2 Effect of olanzapine (0.03 mg/ml) *via* drinking water after 4-week treatment on adipocyte collagen VI in Wistar female rats. Adipocyte was immunohistochemical stained by collagen VI (arrowheads). (A) adipocyte from control animal; (B) adipocyte from olanzapine-treated animals. Original magnification: 100X; scale bar: 100 μm.

3.12 Effect of olanzapine on protein profile of adipose tissue

Silver stain reveals that the protein profile of adipose tissue following olanzapine treatment altered in comparison to the protein profile of the control adipose tissue (figure 3.12.1). At least three protein expressions, i.e. molecular weight approximately 15 KDa, 27 KDa, 30 KDa, respectively, were found increased in olanzapine-treated adipose tissue and two proteins expressions, approximately 21 KDa and 46 KDa, were decreased.

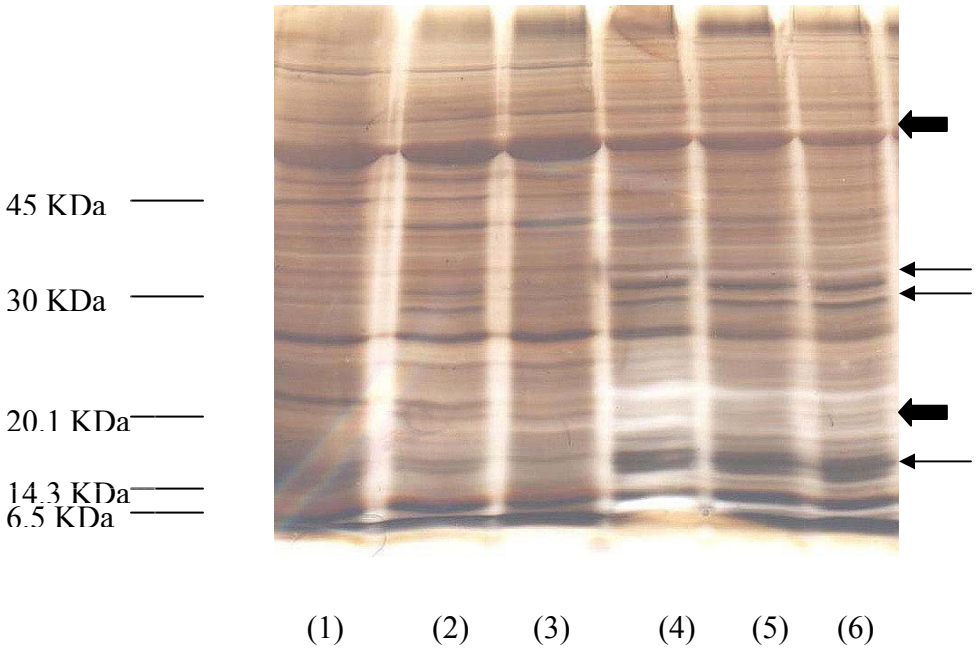


Figure 3.12.1 Effect of olanzapine on protein profile of adipose tissue after 4-week treatment *via* drinking water. (1) — (3): protein profile from control adipose tissues; (4) — (6): protein profile from olanzapine-treated (0.03 mg/ml) adipose tissues.

4 Discussion

4.1 Effect of olanzapine on body weight

In order to investigate the mechanism of olanzapine-induced weight gain, an animal model is required. We adopted a method by administering olanzapine to female Wistar rats *via* drinking water (Leander et al., 2001). The body weight gain was rapidly induced in the first and/or second week (Figure 3.3.1, Figure 3.3.3). Olanzapine administration at 0.03 mg/ml significantly increased body weight after 1 week of treatment and this increase persisted for 4 weeks (Figure 3.3.1). Although olanzapine readily induces weight gain in humans, its effect in rodents appeared less prominent.

Clinical pharmacology studies show that olanzapine has high affinity binding to the following receptors: serotonin $5HT_{2A/2C}$ (K_i = 4 and 11 nM, respectively), dopamine $D_{1\text{-}4}$ (K_i = 11-31 nM), muscarinic M1-5 (K_i = 1.9-25 nM), histamine H_1 (K_i = 7 nM), and adrenergic α_1 receptors (K_i = 19 nM). Olanzapine binds weakly to GABA-A, BZD, and β adrenergic receptors (Ki > 10 mM). Daily dose recommended in clinical treatment is between 5 mg/day to 20 mg/day, roughly 0.1 mg/kg to 0.4 mg/kg per day. The doses of olanzapine used in the present animal study for rats are between 0.3 mg/kg to 6 mg/kg. These doses are reasonably close to the clinical dosage.

It has been reported that female Sprague Dawley rats treated with olanzapine *via* drinking water gained weight (Leander et al., 2001). Other studies have also described effects of olanzapine on weight gain in rats using different drug administration routes. Goudie and colleagues showed that female Wistar rats receiving daily twice injections of olanzapine at a concentration of 4.0 mg/kg exhibited body weight gain after 2 days, and

up to 10 days of treatment (Goudie et al., 2002). Both Arjona and colleagues, Pouzet and colleagues also showed that female Sprague Dawley rats or female Wistar rats showed marked weight gain following olanzapine treatment *via* gavage at 1.2 mg/kg, or at 5.0 and 20 mg/kg (Arjona et al., 2004; Pouzet et al., 2003). Fell and colleagues also found that olanzapine induced significant increase in body weight among female Lister rats treated with doses of 1.0 and 4.0 mg/kg *via* intraperitoneal route (i.p.) (Fell et al., 2004). In contrast to these injection methods, administration of olanzapine *via* drinking water is less invasive and olanzapine is orally administered in humans.

In the present study, olanzapine is capable of increasing weight gain in female Wistar rats. The effect of olanzapine on CD-1 female mice was also tested (unshowed observation). The drug at similar concentration as 0.01 and 0.03 mg/ml *via* drinking water, or at dose as 3 mg/kg *via* gavage, failed to influence weight gain. Moreover, the results from other studies indicated that male rats did not exhibit antipsychotic-induced weight gain (Pouzet et al., 2003). The possible reason might be that physiological period and physical activity are different between male and female rats. But the exact mechanism is still unclear. Therefore, differences in species and gender may affect the outcome of olanzapine's effect.

Results also indicate that a successful drug effect on weight gain was not only related to dose, but also related to how animals were handled. When rats were treated with low dose, i.e. olanzapine solution of 0.003 mg/ml, a significant weight gain cannot be obtained. The effect is therefore dose-dependent. Study of Fell and colleagues showed that olanzapine at a low dose, e.g. 0.5 mg/kg, did not induce weight gain in female Lister rats (Fell et al., 2004). In one experiment of the present investigation, the rats were

treated at 0.02 mg/ml or 0.04 mg/ml, approximately 2.4 mg/kg to 6 mg/kg, and significant body weight gain was not obtained. Neither food nor water intake were increased (data not shown). This was probably due that the applied stressful treatment was too stressful to the animals. During the drug treatment period, the animals were fasted twice a week, 12 hours each time, and blood samples were collected each week. Animals had to be immobilized for at least 15 minutes during the blood collection. This excessive treatment might have been overly stressful to these animals. It is indicated that severe and prolonged stress impairs normal physiology and nullified the olanzapine-induced weight gain. Therefore, in the study on effect of olanzapine on weight gain, any stressful treatment to the animals should be avoided.

The rats also showed marked individual difference in weight gain. In the dose dependent experiments, a large within-group variance was always observed which diminished the power of analysis of effect of olanzapine on weight gain in the whole group. After analysis of the distribution of the weight gain in the treated rats, in a concentration range (0.003 mg/ml — 0.03 mg/ml), it was determined that 9/20 (45%) of the animals gained more weight. After 1-week treatment, the increase was evident and persisted during the period of treatment. The remainder, e.g. 11/20 (55%) rats, obtained had no or only moderate weight gain. Clinical reports have shown that only a portion, about 30% of olanzapine-treated patients gain weight (Green, 2000). Our animal data seem comparable to that of the clinical observations.

A number of studies have shown that body fat increase is a determining factor of weight gain-associated mortality (Eder et al., 2001). In the present study, olanzapine fed *via* drinking water at 0.006 and 0.01 mg/ml significantly increased abdominal fat

deposition, and this effect was also observed in 0.03 mg/ml treated group. However, due to the large variance in this group, there was no difference compared to the control group. Other laboratories also observed a large variance in the olanzapine-treated female rats (Daniels et al., 2003; Fell et al., 2004). Clinical studies demonstrated that olanzapine-induced weight gain in patients was accompanied with an increase in body fat (Eder et al., 2001; Murashita et al., 2005; Ryan et al., 2004). An analysis of visceral fat distribution pattern reveals that patients after olanzapine treatment had a higher level of intra-abdominal fat than that of control subjects. Interestingly, there was no significant difference in subcutaneous fat (Ryan et al., 2004). The present data indicated that olanzapine-induced body weight gain in rats was also attributable to an increase in both subcutaneous and intra-abnormal adipose tissues.

Olanzapine significantly increased food and water intake during the test period. However, significant increase in food consumption was observed only in the first week. No consistent increase in food intake was observed during the whole period of treatment. Nevertheless, the finding of this study is consistent with a number of clinical observations and animal studies that olanzapine induces short-term hyperphagia in both patients and rodents. Two clinical studies indicated that although inconsistent with time course of treatment, increase in caloric intake was observed in treated patients, and there was no change in physical activity of these patients (Eder et al., 2001; Gothelf et al., 2002). In rodent studies, a short-term olanzapine-induced hyperphagia was observed by different groups, although this effect may vary with dose, methods of administration and measurement (Arjona et al., 2004; Fell et al., 2004; Hartfield et al., 2003; Kaur et al., 2002; Lee et al., 2002; Pouzet et al., 2003; Thornton-Jones et al., 2002). Increase in

caloric intake without accompanied increase in physical activity would cause body weight gain. The enhancement on feed efficiency seen in the overweight rats in the present study may explain at least in part how drug-induced weight gain is related to increased food intake without marked behavior change. Increase in water intake in these treated rats is comparable to the study of Fell and colleagues (Fell et al., 2004). Serotonin is well recognized in the control of food intake (Arjona et al., 2004) and histamine is a well-known regulator of water intake (Casey et al., 2001; McIntyre et al., 2001a; McIntyre et al., 2001b). Olanzapine exhibits broad a pharmacological profile. The blockade of 5-HT_{2C} and H_1 receptors by olanzapine may be responsible for the change of feeding behavior.

4.2 Effect of olanzapine on blood levels of glucose, triglyceride and cytokines

In the present study, olanzapine exhibited some effects on the blood glucose level, but not on the triglyceride levels. These results are inconsistent to the clinical data. Different clinic reports showed that after 14 weeks to 25 months treatment with olanzapine, around 11% to 44% of patients were hyperglycemic (Melkersson et al., 2004; Newcomer, 2004; Wirshing et al., 2002), while around 39% to 62% of patients were hypertriglyceridemic (Casey, 2004; Melkersson et al., 2000; Melkersson et al., 2004; Wirshing et al., 2002). A retrospective analysis showed that the mean time to peak triglyceride levels in treated patients was 10.0 months (Casey, 2004). A similar result from other lab also showed that olanzapine did not induce hyperglycemia in male Sprague-Dawley rats (Patel et al., 2004). Therefore, the effect of olanzapine on blood glucose and triglyceride levels may be influenced by the period of treatment. The small

number of animals in the present series of experiments may also limit the power of analyses. It is possible that the apparent discrepancy may be due to species difference, insufficient treatment duration, and sample size.

Only blood insulin levels, not leptin and TNF-α levels, were found to be significantly affected by olanzapine, but the effect was not dose-dependent. Both insulin and leptin levels were found positively correlated with high weight gain in the olanzapine-treated animals. Insulin, the most important hormone involved in regulating glucose metabolism, has been reported to be increased in olanzapine-treated patients even without an increase in glucose (Eder et al., 2001). Significant increase in incidence of pancreatitis, i.e. 33%, was found associated with olanzapine treatment (Newcomer, 2004). A recent *in vitro* finding suggests that olanzapine may affect insulin release from pancreatic β cells (Melkersson, 2004). But to date, there is no evidence to show that an increase in insulin level is related to olanzapine's effect on the pancreas. The present experiment did not show a significant correlation between weight gain and the blood insulin levels, nor between the increase in adipose masses and insulin. In contrast, the level of leptin was highly correlated with both body weight gain and adipose tissue masses. Since leptin is synthesized from adipocyte, in contrast to insulin which is secreted from pancreatic cells, such a correlation can be expected. A positive correlation between leptin and BMI was also reported (Atmaca et al., 2003). Higher rates of diabetes have been reported in olanzapine-treated patients and many olanzapine-treated patients have recovered from diabetes after discontinuing of the medication (Melkersson et al., 2004). It is quite possible that an insulin and leptin resistance might be induced by olanzapine.

The role of TNFα in olanzapine-induced weight gain has drawn some attention but little is known. TNFα and its receptors are increased in clozapine–treated patients (Pollmacher et al., 1996). An increase in TNFα receptor levels was also found in olanzapine-treated patients (Schuld et al., 2000). Olanzapine is chemically very similar to clozapine. The circulatory TNFα receptor is thought to be a sensitive marker for TNFα activity. Many studies have shown that TNFα may induce insulin resistance, leading to impaired glucose metabolism (McIntyre et al., 2001a; Zimmermann et al., 2003). Both TNFα and its receptors can be synthesized by adipocytes. Blood TNFα was not correlated to body weight change in the present experiment. Results do not support the idea that olanzapine affect the involvement of TNFα. However, the large within-group variation makes any conclusion difficult. The methodology has some limitation. In order to reassess the role of TNFα in olanzapine-induced weight gain, improved ELISA or larger number of animals is required. TNFα receptor levels were not examined in the present study. Clearly, further study is needed in order to delineate the role of TNFα system in olanzapine-induced weight gain.

4.3 Effect of olanzapine on adipocyte

That olanzapine dramatically altered the morphology of adipose tissues was a serendipitous finding. The effect was consistent and reproducible in most olanzapine-treated animals. This is the first observation that olanzapine not only increased the deposition of adipose tissue, but also changed the morphology of adipose tissue. Such morphological change was detected even in olanzapine-treated rats without obvious weight gain. It was thought that adipocytes differentiation might be influenced by

olanzapine. Due to the difficulties in identifying the structure of adipocyte at early development stage, marker proteins representing adipose differentiation was assessed. It has been found that morphology changes in adipocyte differentiation are accompanied with alterations in extracellular matrix components. As one component of extracellular matrix, the α chain 2 of type VI collagen is known to be an early marker for differentiation of adipocytes. Collagen VI levels are increased several-fold and persisted after initiation of adipose conversion (Klaus, 2001, Nakajima et al., 2002). In the present study, the immunohistochemical stain revealed that indeed extracellular collagen VI expression of adipocytes was dramatically enhanced. The presence of such collagen VI change suggests that an enhancement of adipocyte differentiation was stimulated by olanzapine treatment. The increase in adipose tissue mass in obesity includes both hyperplasia and hypertrophy. The result of collagen stain indicates that olanzapine-induced weight gain is probably in part due to the stimulation of adipocyte differentiation.

It is interesting to note that although the increase of adipose tissue mass was seen in both abdominal and subcutaneous, such a morphological change was primarily constrained in subcutaneous fat deposition. The different effects of olanzapine on regional adipose tissues are unclear.

The protein profiles in olanzapine-treated adipose tissues were also substantially altered. It has been shown that during the stage from adipoblasts to mature lipid-containing adipocytes, a large set of enzymes and proteins emerge and are involved in the different developmental stages (Klaus, 2001). Several adipose proteins were found substantially changed both increase and decrease by olanzapine. These proteins have not

been identified. It remains to be established whether these proteins are involved in or a result of adipose differentiation. Nevertheless, the result indicates that dramatic change in metabolism in adipose take place following chronic treatment of olanzapine.

In the present *in vitro* experiment using cultured differentiated 3T3-L1 adipocytes, olanzapine did not affect the viability and proliferation of the cells, as revealed by MTT test. Olanzapine also did not change the cellular triglyceride level in treated 3T3-L1 adipocytes. Upon growth arrest in the presence of fetal bovine serum and with the stimulation of dexamethasone (Dex), isobutylmethylxanthine (IBMX) and insulin, triglyceride starts to accumulate in 3T3-L1 cells and to form cytoplasmic lipid vesicles (Croissandeau et al., 2002). The lipid accumulation is an event which appears when the immature adipocyte differentiates to mature adipocyte (Klaus et al., 2001). The unchanged cellular triglyceride levels following olanzapine treatment indicated that olanzapine did not directly affect adipocytes differentiation *in vitro*. It is hypothesized that some endocrine factors, which altered by olanzapine, may mediate the effect of olanzapine on adipose tissue *in vivo*.

The deposition of white adipose tissues is known to be dependent on gender. In humans, abdominal fat mass is predominant in the male, while subcutaneous fat mass is more prominent in the female (Klaus et al., 2001). Increased abdominal adipose tissue, especially visceral abdominal adiposity can increase insulin resistance and contribute to hyperglycemia and diabetes (Haupt et al., 2001). In the present study, treated subjects with morphological change in the subcutaneous part were all female rats. It is yet to be determined whether olanzapine exerts the same effect on adipose differentiation in male

rats or mice. It is also very interesting to know whether morphological changes of adipocytes would take place in patients treated with olanzapine.

4.4 Conclusion

In the present study, an animal model was employed to investigate the mechanism of olanzapine-induced weight gain. Insulin and leptin were found changed in association to olanzapine-induced weight gain. The morphological change and increase in fat deposition contributed towards the weight gain during olanzapine treatment. However, there are still many questions to be answered.

Firstly, it would be very interesting to examine how olanzapine influences feeding behavior, which may be related to its pharmacological profile. The accurate feeding patterns should be monitored, including the frequency, duration time, and meal size. Meanwhile, the levels of neurotransmitters in the brain should be tested to determine which one is correlated to the change in feeding behavior.

Secondly, although insulin and leptin appear to be involved in olanzapine-induced weight gain, the mechanism involved in olanzapine-induced hyperinsulinemia is still unknown. Since the prevalence of diabetes is significantly higher in olanzapine-treated patients, it is necessary to understand how olanzapine influences pancreas and the insulin signal pathway.

Further research is required to determine the molecular mechanism of olanzapine-stimulated adipocyte differentiation. It would be interesting to know how olanzapine acts on adipocytes and why olanzapine primarily acts on subcutaneous adipocytes. It is known that preadipocyte proliferation, differentiation and their interrelations are very complex. It would be interesting to know whether hormones, neural system, and paracrine factors are

involved, and how the effect is related to gender. An animal model for studying the olanzapine-induced weight gain will be useful in uncovering the underlying mechanism of weight gain induced by atypical antipsychotics. Clearly, it is important to answer that question whether the results from the animal studies can be translated to the clinical situation.

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Appendix List of chemicals and reagents

Chemicals	Suppliers
Acetic acid	BDH
Acetonitrile	BDH
ATP	Sigma
Bovine serum albumin(BSA)	Sigma
Chloral hydrate	Sigma
Diaphorase	Sigma
DAB (3, 3'- Diaminobenzidine)	Sigma
Dexamethasone (Dex)	Sigma
D-glucose	Sigma
Disodium phosphate anhydrous	EMD
DMEM	Sigma
EDTA	Sigma
Ethanol	Chemical Alcohol Inc.
Eosin	Ricca chemical Co.
Extravidin-horse radish peroxidase	Sigma
Fetal calf serum (FBS)	Hyclone
Glycerol kinase	Sigma
Glycerol -1- phosphate dehydrogenase	Sigma
HEPES	Sigma
Mayer's Hematoxylin	Sigma
Hydrogen peroxide	EMD
Isobutylmethylxanthine (IBMX)	Sigma
Lipoprotein lipase	Sigma
New born calf serum (NBCS)	Sigma
Olanzapine	Eli Lilly
Paraformaldehyde	Sigma
Penicillin	Sigma
Phosphoric acid	Fisher
poly-L-lysine	Sigma
2-(p-iodophenyl)-3-p-nitrophenyl-5-phenyltetrazolium chloride (INT)	Sigma
Potassium chloride	EMD
Potassium phosphate	EMD
Sodium azide	Aldrich chemical
Sodium bicarbonate	EMD
Sodium hydroxide	Sigma
Sodium Phosphate Dibasic Anhydrous	EMD
Sodium pyruvate	Sigma
Streptomycin	Sigma
Sulphuric acid	BDH
Tetramethylbenzidine dihydrochloride (TMB)	Sigma
Triethylamine	Sigma

Trypsin	Sigma
Tween 20	Sigma
xylocaine Jelly 2%	Astra Pharma Inc.
xylene	EMD

ELISA kit (leptin, insulin and TNFα)

Leptin:	Goat anti-mouse leptin antibody	R&D Systems
	Recombinant rat leptin	
	Biotinylated goat anti-mouse leptin antibody	
Insulin:	Mouse monoclonal insulin antibody	Mercodia
	Rat insulin standard	
	Peroxidase conjugated mouse monoclonal anti-insulin	
TNFα:	Goat anti-mouse TNFα antibody	Peprotech
	Recombinant rat TNFα	
	Biotinylated goat anti-mouse TNFα antibody	

Goat anti- mouse collagen VI antibody	Santa Cruz
Peroxidase-conjugated rabbit anti goat IgG	Sigma